DEPARTMENT OF NEUROLOGY SERIES OF REPORTS NO 84, 2006

PETRI KEROKOSKI

Regulation of Cyclin-Dependent Kinase 5 (Cdk5)

With Special Emphasis on Changes Occurring During Neuronal Cell Death

Doctoral dissertation

To be presented with assent of the Medical Faculty of the University of Kuopio for public examination in Auditorium L1, Canthia building, University of Kuopio, on Friday 30th June 2006, at 12 noon

Department of Neurology, University of Kuopio
Department of Neurology, Kuopio University Hospital

Distributor:
Department of Neurology
University of Kuopio
P.O. Box 1627
FIN-70211 Kuopio
FINLAND
Tel. +358 17 162 682
Fax +358 17 162 048

Author's address:
Department of Neuroscience and Neurology
University of Kuopio
P.O. Box 1627
FIN-70211 Kuopio
FINLAND
Tel. +358 50 376 2133
Fax +358 17 162 048
E-mail: Petri.Kerokoski@luukku.com

Supervisors:
Professor Tuula Pirttilä, MD, PhD
Department of Neurology
Kuopio University Hospital

Professor Hilkka Soininen, MD, PhD
Department of Neurology
Kuopio University Hospital

Reviewers:
Docent Marc Baumann, PhD
Protein Chemistry/ Proteomics Unit
and the Neuroscience Program
Biomedicum Helsinki
University of Helsinki

Docent Irma Holopainen, MD, PhD
Department of Pharmacology and Clinical Pharmacology
University of Turku

Opponent:
Docent Urmas Arumäe, PhD
Institute of Biotechnology
University of Helsinki

ISBN 951-781-376-7
ISBN 951-27-0214-2 (PDF)
ISSN 0357-6043

Kopijyvä
Kuopio 2006
Finland

Kerokoski, Petri. Regulation of Cyclin-Dependent Kinase 5 (Cdk5). With Special Emphasis on Changes Occurring During Neuronal Cell Death. Series of Reports, No 84, Department of Neurology, University of Kuopio 2006, 98 p.
ISBN 951-781-376-7
ISBN 951-27-0214-2 (PDF)
ISSN 0357-6043

ABSTRACT

Neuronal loss occurs in several neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease, as well as following acute insults, such as brain ischemia and trauma. In general, cell death can be categorized into two main types, apoptosis and necrosis, which are differentiated by distinct morphological and biochemical features. Cell death of neurons possesses special characteristics that are still inadequately understood. Deregulation of the cyclin-dependent kinase 5 (cdk5) has been suggested to have several consequences that might contribute to the course of neuronal death in both acute and chronic conditions. This deregulation could involve proteolytic cleavage of the cdk5 activator protein p35 to a p25-fragment by the calcium-activated calpain-protease. Nevertheless, there are wide gaps in our understanding on the regulation of cdk5, which hampers the evaluation of the possible involvement of cdk5 in neuronal death.

The aim of this study was to investigate the regulation of cdk5, in particular during the course of neuronal cell death, and also to evaluate the possible relationship between cdk5 and phosphorylation of the microtubule-associated protein tau. When cultured rat hippocampal neurons were subjected to various apoptosis-inducing compounds, one characteristic response was a decrease in the protein levels of p35, p25, and cdk5 as well as a reduction in the cdk5 activity and tau phosphorylation. Toxicity caused by the neurotransmitter glutamate, or by calcium ionophores, was associated with a rapid calpain-mediated cleavage of p35, and an increase in the cdk5 activity. Unexpectedly, tau phosphorylation was concomitantly decreased. Both N-methyl-D-aspartate (NMDA) and non-NMDA classes of glutamate receptors were able to induce calpain-mediated p35 cleavage in the cultured neurons. However, the NMDA receptors predominantly mediated the effect of glutamate in neurons under normal culture conditions. A significant proportion of the p35 protein was detected in a phosphorylated state in rat brain tissue. Phosphorylation of p35 appeared to affect its proteolytic degradation by the proteasome, as well as the calpain-mediated cleavage of p35 to p25.

In conclusion, the regulation of cdk5 is altered during the process of neuronal death. The neurotransmitter glutamate could be an especially relevant inducer of the putative cdk5-associated mechanisms of cell death. However, the changes in cdk5 during neuronal death do not seem to be invariably linked to the phosphorylation status of tau protein.

National Library of Medicine Classification: QU 57, QU 135, QU 143, QU 325, QU 375, QY 60.R6, WL 102.5, WL 104, WL 314, WL 359
Medical Subject Headings: adenosine triphosphate; brain chemistry; calpain; cell death; cells, cultured; cyclin-dependent kinase 5; cysteine endopeptidases; dipeptides; enzyme activation; enzyme inhibitors; glutamic acid; hippocampus/cytology; hippocampus/drug effects; nerve tissue proteins; neurodegenerative diseases; neurons/drug effects; neurons/metabolism; phosphorylation; rats; receptors, glutamate; tau proteins

To Elina, Elias, and Martta

ACKNOWLEDGEMENTS

This work was carried out in the Department of Neuroscience and Neurology of the University of Kuopio during the years 2000-2005.

I am most grateful to my supervisors Professor Tuula Pirttilä and Professor Hilkka Soininen for their enthusiastic guidance and professional teaching, as well as for their continuous support and encouragement.

I warmly thank my co-authors Antero Salminen and Tiina Suuronen for their contributions to the study. I am especially indebted to Antero for always being eager to share his knowledge, and for providing me with such excellent facilities.

I want to express my gratitude to Docent Marc Baumann and Docent Irma Holopainen, the official reviewers of this thesis, for their constructive criticism and suggestions for improving the manuscript.

I thank Nilla Nykänen, Tuija Parsons, Mari Tikkanen, and Tuula Toivanen for their kind assistance. Special thanks belong to Sari Palviainen, Esa Koivisto, and Marja-Leena Soininen for all the help I have received. I also thank the personnel of the National Laboratory Animal Center of the University of Kuopio. In addition, I thank all the persons involved in teaching me neuroscience.

Many thanks to Erkki Kuusisto and Heikki Tanila for critical reading of the manuscripts and for their valuable thoughts, as well as to Ewen MacDonald for revising the language of this thesis.

My sincere thanks belong to the entire personnel of the Department of Neuroscience and Neurology for providing a uniquely pleasant atmosphere to carry out this work. Especially, I feel privileged for having being able to work with such fine companions in the Antero's laboratory during these years. I owe my deepest gratitude to all of them.

Very special thanks to the current and former graduate students in the department for all the joyful time shared. I am grateful to each one of them for their companionship and friendship. Especially I thank Erkki Kuusisto for the almost never-ending discussions covering all of the major aspects of life. I also wish to thank all the members of our "sähly" team for making Thursday the most pleasant working day of the week.

I am grateful to all my relatives and friends for their support and encouragement. I owe my warmest thanks to my parents Saini and Simo, and to my brothers Olli and Marko and their families, as well as to my late brother Esa, for being my foundation.

Finally, my dearest thanks to my beloved wife Elina for her love and support which encouraged me during the critical times. I thank Elias and Martta for introducing me to a completely new level of happiness.

This study was financially supported by the Health Research Council of the Academy of Finland, EVO grants (5510, 5142, 5152, 5165, 5772708, 5772725) of the Kuopio University Hospital, the University of Kuopio, the Finnish Cultural Foundation of Northern Savo, the Emil Aaltonen Foundation, the Kuopio University Foundation, the Research and Science Foundation of Farmos, and the Finnish Cultural Foundation.

Kuopio, March 2006

Petri Kerokoski

ABBREVIATIONS

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
Apaf-1	apoptosis protease-activating factor-1
APP	amyloid precursor protein
BSA	bovine serum albumin
CAK	cdk-activating kinase
Cdk5	cyclin-dependent kinase 5
CIP	cdk5 inhibitory peptide
CKI	casein kinase I
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
Con A	concanavalin A
CTZ	cyclothiazide
DARPP-32	dopamine and cAMP-regulated phosphoprotein, 32 kDa
DHPG	3,5-dihydroxyphenylglycine
DR	death receptor
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FBS	fetal bovine serum
FTDP-17	frontotemporal dementia with parkinsonism linked to chromosome 17
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
HNE	4-hydroxynonenal
IP_3	inositol 1,4,5-triphosphate
JNK3	c-Jun N-terminal kinase 3
LDH	lactate dehydrogenase
LTP	long-term potentiation
MAP	microtubule-associated protein
MAPK	mitogen-activated protein kinase
MEF2	myocyte enhancer factor 2
MEK1	MAPK kinase 1
mGluR	metabotropic glutamate receptor
MOPS	3-[N-morpholino]propanesulfonic acid
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NBQX	2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide
NF	neurofilament
NFT	neurofibrillary tangle
NMDA	N-methyl-D-aspartate
NOS	nitric oxide synthase
PBS	phosphate-buffered saline
PCD	programmed cell death
PD	Parkinson's disease
PI3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PP-1	protein phosphatase-1

PTP	permeability transition pore
RasGRF2	Ras guanine nucleotide releasing factor 2
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOD1	superoxide dismutase 1
TNF	tumor necrosis factor
VSCC	voltage-sensitive calcium channel

CONTENTS

1. INTRODUCTION

Cell death occurs throughout the lifespan of multicellular organisms. Cell death is essential for many processes e.g. development, maintenance of tissue homeostasis, and for removal of damaged cells. Turnover of cells provides a means for regeneration in a number of different tissues. The existence of neuronal stem cells in adult brains indicates that also neurons can be replaced. However, the sophistication of the nervous system originates from the complex cellular architecture and functional properties established over a long period of time, starting well before birth. These features cannot be fully achieved by cells born later in life. For this purpose, the neuronal cells responsible for the elegance of the nervous system have a long lifespan allowing the cells to carry out their responsibilities throughout the life of the organism.

Even though neurons are considered to be irreplaceable, there is significant neuronal death during the development of the nervous system, and death of neurons continues at low rate also throughout adulthood. This cell death does not compromise the functionality of the nervous system, as neurons are originally produced in excess, and the functions of the lost cells are often compensated by plastic changes in the remaining neurons. In some pathological conditions cell death can take place to such extent that the functionality of the system becomes impaired. Neuronal death can occur suddenly following acute insults such as ischemia or traumatic brain injury. Pathological cell death can also be slow and progressive as occurs in neurodegenerative disorders like Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). The symptoms of these disorders often cause a major decline in independent functional capacity as well as in the quality of life, and lead to a long-term burden to the patients and their relatives. Furthermore, treatment of patients suffering from these disorders consumes a significant amount of health care resources.

Research on the molecular mechanisms of cell death increases our understanding of basic biological processes, and provides a foundation for efforts aiming to prevent pathological cell death. As neurons generally cannot be replaced, finding ways to prevent neuronal death is of particular interest. The neuronal cells possess several

exceptional features, and therefore knowledge on the death of other cell types cannot be directly applied to neurons. Also neuronal death in different pathological conditions differs to such extent that understanding of the molecular events contributing to cell death in one disease cannot be extrapolated to other neurological conditions. The amount of information that can be obtained by studying neuronal death in human patients is restricted. Experimental modeling of various neuropathological conditions in laboratory animals, as well as in tissue and cell cultures, represents one possibility to considerably extend our knowledge on the cascades of molecular events ultimately leading to pathological neuronal death. The cyclin-dependent kinase 5 (cdk5) is one example of an enzyme that has been implicated in the molecular pathways underlying neuronal death. Cdk5 plays a pivotal role in the normal biology of neurons, however in some neuropathological conditions cdk5 has been suggested to become deregulated, and this may have pathological consequences. A detailed understanding on the regulation and functional significance of this enzyme is needed to dissect its possible contribution to pathological events.

2. REVIEW OF THE LITERATURE

2.1. Molecular mechanisms of neuronal cell death

Cell death is often divided into two main types i.e. apoptotic and necrotic cell death. This distinction is classically made on the basis of morphological features, although also certain biochemical hallmarks are associated with different types of cell death (Syntichaki and Tavernarakis 2003, Yuan et al. 2003). Apoptosis is an ordered and active "suicide" form of cell death, whereas necrosis represents a more uncontrolled and passive outcome of failed cell homeostasis (Orrenius et al. 2003, Yuan et al. 2003). Frequently cell death occurs in a manner that cannot be classified as being purely apoptotic or necrotic. Sometimes the features of cell death differ so extensively from these two main types, that additional classifications are used. For example, destruction of the cell by its own lysosomal system has been suggested to represent a special form of cell death, i.e. autophagic cell death (Yuan et al. 2003, Assuncao Guimaraes and Linden 2004). Excitotoxic cell death is a form of cell death that is characterized by its distinct cell death-inducing stimulus. Neuronal cell death as a result of excitotoxicity is of particular importance, since this kind of cell death is involved in many pathological conditions of the nervous system (Lipton and Rosenberg 1994).

2.1.1. Necrotic cell death

Necrotic cell death has been traditionally regarded as a chaotic breakdown of cells occurring under intolerable conditions. However, recent studies have revealed, that similarly as previously observed in apoptosis, also necrosis seems to involve a limited number of biochemical cascades, although there is no molecular machinery especially evolved for this purpose (Syntichaki and Tavernarakis 2003, Yuan et al. 2003). Rather the cell death-inducing stimuli lead to destabilization of normal cellular activities. Once disturbed, these normal physiological processes become destructive and promote a cascade of events leading to cell death (Syntichaki and Tavernarakis 2003, Yuan et al.

2003). Necrosis usually takes place as a result of severe injury. Nevertheless, there are also several examples of necrotic cell death occurring during the normal course of development and tissue regulation ("programmed cell death", PCD) (Proskuryakov et al. 2003, Assuncao Guimaraes and Linden 2004, Edinger and Thompson 2004).

The morphological characteristics of necrosis include clumping of chromatin without any marked changes in its distribution, swelling of mitochondria, and local disruption of cellular membranes. Subsequently there is pronounced disintegration of organelles and membranes, disappearance of chromatin, and cell lysis (Lossi and Merighi 2003, Yuan et al. 2003). Generally necrosis involves a large number of cells that are often grouped together. As the contents of the cells are released into the surrounding tissue, necrosis is associated with an inflammatory response. The remains of the necrotic cells are eventually removed by phagocytosis (Lossi and Merighi 2003).

Perturbation of cellular ion homeostasis, especially calcium homeostasis, has been closely linked with necrotic cell death. However, calcium-involving mechanisms are not specific for necrosis, as calcium is commonly associated with apoptosis as well (Leist and Nicotera 1999, Syntichaki and Tavernarakis 2003, Yuan et al. 2003). Normally calcium functions as a ubiquitous intracellular messenger, controlling the activity of a number of enzymes, ion channels, etc. The level of intracellular calcium is actively kept low when compared to the extracellular space and to the calcium stores within the endoplasmic reticulum (Alberts et al. 2002, Orrenius et al. 2003, Syntichaki and Tavernarakis 2003). Activation of calcium channels on the plasma membrane or in the endoplasmic reticulum leads to local elevations in the level of intracellular calcium. The mitochondria can buffer the intracellular calcium level by taking up the cation when the intracellular calcium concentration is high (Alberts et al. 2002, Syntichaki and Tavernarakis 2003, Duchen 2004). However abnormally intense calcium-mediated signaling can lead to pronounced activation of several cell death-associated enzymes such as proteases (calpain), lipases (phospholipase A_2), nitric oxide synthase (NOS), kinases (protein kinase C), and phosphatases (calcineurin). Calcium also can evoke cell death via its effects on the mitochondria (Leist and Nicotera 1999, Orrenius et al. 2003, Wu et al. 2004).

Several proteases are known to be involved in necrotic cell death. Induction of the calcium-activated calpain proteases frequently occurs during necrosis (Wang 2000, Syntichaki and Tavernarakis 2003). One important group of necrosis-associated calpain targets are the cytoskeletal proteins, such as αII-spectrin, the microtubule-associated protein tau, and tubulin (Wang 2000, Rami 2003). During necrosis, also the lysosomal cathepsin proteases can leak into the cytosol, possibly due to calpain-mediated destabilization of lysosomal membrane integrity, or following damage to the lysosomal membranes by free radicals (Nakanishi 2003, Syntichaki and Tavernarakis 2003). Also the caspase proteases, traditionally associated with apoptotic cell death, can be activated during necrosis. This possibly occurs due to leakage of caspase activating factors from damaged mitochondria, or by calpain or cathepsin mediated cleavage. Indeed, a complex cascade of protease activation may take place also during necrosis (Syntichaki and Tavernarakis 2003).

The mitochondria are the major source of ATP within the cell. Disturbed mitochondrial function, such as that commonly occurring during necrosis, leads to depletion of energy, as well as to production of reactive oxygen species (ROS). Leakage of electrons from the mitochondrial electron transport chain promotes formation of ROS such as superoxide ions (O_2^-) and H_2O_2 (Proskuryakov et al. 2003, Duchen 2004). Another cellular ROS is nitric oxide, a diffusible free radical second messenger, produced by the enzyme, NOS. Importantly, the neuronal NOS isoform (nNOS) is regulated in a calcium-dependent manner (Davis et al. 2001, Duchen 2004). Excessive levels of ROS can induce damage to proteins, lipids, and nucleic acids. In mitochondria, ROS, especially when combined with an elevated level of intracellular calcium, contribute to cell death by promoting the opening of a non-specific mitochondrial pore, called the permeability transition pore (PTP) (Orrenius et al. 2003, Duchen 2004, Gunter et al. 2004). Opening of the PTP results in mitochondrial depolarization, and subsequently to increased permeability of the outer mitochondrial membrane such that even large for protein molecules can leak into the cytosol (Gulbins et al. 2003, Duchen 2004).

2.1.2. Apoptotic cell death

Apoptosis was originally described as cell death characterized morphologically by cellular shrinkage in the presence of maintained organelle integrity, condensation of nuclear chromatin, plasma membrane blebbing, cellular fragmentation, and phagocytosis of the remaining apoptotic bodies by neighboring cells (Kerr et al. 1972). Later, also certain biochemical hallmarks of apoptosis, such as activation of caspase-proteases and oligonucleosomal cleavage of chromatin, have been described (Zakeri and Lockshin 2002, Friedlander 2003, Edinger and Thompson 2004). The majority of PCD occurs by apoptosis, and it is frequently detected also in pathological situations (Friedlander 2003, Lossi and Merighi 2003).

Apoptosis is an active process requiring energy and usually also synthesis of new proteins. Apoptosis can be triggered be several factors e.g. external death-promoting factors, loss of external factors needed for survival, or signs of internal damage (Creagh et al. 2003, Benn and Woolf 2004). The initial intracellular events involved in transmitting and modulating the apoptosis-promoting signals vary considerably depending on many factors as the initial stimulus and the cell type. Changes in calcium signaling, ROS production, kinase activities, gene expression, and mitochondrial function commonly take place (Friedlander 2003, Benn and Woolf 2004). Nevertheless, the downstream cellular machinery which integrates the pro-apoptotic and anti-apoptotic signals, and subsequently executes the apoptotic program, is quite stereotypic and rather well conserved during evolution (Creagh et al. 2003, Friedlander 2003, Lossi and Merighi 2003, Yuan et al. 2003). Once the critical executioners of apoptosis have been activated, the process continues by ordered degradation of DNA into oligonucleasomal fragments, and disassembly of cellular structures. Since the remains of the cells are removed by phagocytosis and the cellular contents are not released to the surrounding tissue, there is no inflammation associated with apoptotic cell death (Creagh et al. 2003, Friedlander 2003, Assuncao Guimaraes and Linden 2004).

Activation of the caspase family of proteases (cysteine-dependent aspartate-specific proteases) is regarded as being an important step in the commitment to apoptotic cell

death (Creagh et al. 2003, Sprick and Walczak 2004). The caspases constitute a step where the best established apoptosis-associated biochemical pathways converge, although it should be noted that the caspases can also be bypassed during the course of apoptosis (Orrenius et al. 2003, Assuncao Guimaraes and Linden 2004). The caspases exist in the cytosol as inactive zymogens (procaspases), and the upstream initiator caspases (caspase-2, -8, -9, and -10) are activated as a result of pro-apoptotic signaling. Once activated, the initiator caspases activate the downstream effector caspases (caspase-3, -6, and -7) in a cascade-like manner (Creagh et al. 2003, Sprick and Walczak 2004). The effector caspases themselves cleave key enzymes, structural proteins, and regulatory proteins, in addition to activating additional destructive enzymes (Earnshaw et al. 1999, Creagh et al. 2003). The most widely recognized pathways leading to caspase activation, e.g. the mitochondrial pathway activating caspase-9, and the death receptor pathway involving caspase-8, are briefly described below.

The mitochondria have a key role in integrating pro- and anti-apoptotic signals from a variety of sources. The balance between pro-apoptotic (e.g. Bax, Bak, Bid, Bad, Bim) and anti-apoptotic (e.g. Bcl-2 and Bcl-x_L) members of the Bcl-2 family of proteins contributes to the control of the mitochondrial PTP (Bortner and Cidlowski 2002, Akhtar et al. 2004, Benn and Woolf 2004, Green and Kroemer 2004). Once the PTP is opened, there is an increase in the permeability of the outer mitochondrial membrane, and release of several pro-apoptotic proteins from the mitochondria (Gulbins et al. 2003, Hajnoczky et al. 2003, Benn and Woolf 2004). When cytochrome c, a member of the mitochondrial electron transport chain, is released into the cytosol, it forms a complex called the apoptosome together with apoptosis protease-activating factor-1 (Apaf-1), procaspase-9, and dATP. This complex activates caspase-9, leading to induction of downstream caspases (Friedlander 2003, Gulbins et al. 2003, Benn and Woolf 2004). Apoptosis induced by many stress stimuli involve mitochondria at an early stage. The stimuli can alter the balance between pro- and anti-apoptotic Bcl-2 family proteins, by affecting gene expression or by inducing changes at the protein level (e.g. by protein-protein interactions, altered phosphorylation, or by proteolysis) (Gulbins et al. 2003, Akhtar et al. 2004, Benn and Woolf 2004). Several stress stimuli

can also increase the concentration of intracellular free calcium, as frequently occurs during apoptosis. Excessive influx of calcium to the mitochondria promotes opening of the PTP, disruption of mitochondrial membrane potential, and the generation of ROS (Hajnoczky et al. 2003, Orrenius et al. 2003).

Some members of the tumor necrosis factor (TNF) receptor superfamily are called death receptors (DRs) due to their ability to activate pro-apoptotic intracellular signaling. These receptors include Fas, TNFR1, DR3, DR4, DR5, and DR6 (Curtin and Cotter 2003). Once the ligand binds to its binding site, the receptors oligomerize, and recruit an intracellular signaling complex, promoting activation of procaspase-8 (Curtin and Cotter 2003, Benn and Woolf 2004, Sprick and Walczak 2004). In some cell types, the activation of the caspase-8 is sufficient to activate downstream effector caspases. In other cell types, activation of caspase-8 leads to cleavage of the Bcl-2 family member Bid, which promotes apoptosis through the mitochondrial apoptotic pathway (Scaffidi et al. 1998, Curtin and Cotter 2003, Benn and Woolf 2004, Sprick and Walczak 2004). Activation of death receptors is a common trigger of PCD in different tissues. With respect to neuronal cells, up-regulation of Fas-ligand expression occurs in response to a deficiency of neurotrophic factors, and Fas activation seems to be obligatory for apoptosis induced by trophic factor deprivation in several types of neuronal cells (Le-Niculescu et al. 1999, Raoul et al. 1999).

Although necrosis and apoptosis have been classically regarded as distinct and separate processes, there does appear to be a partial overlap in the molecular mechanisms underlying these two types of cell death. Whether the partly shared biochemical processes give rise to an apoptotic or necrotic morphological pattern, may be determined by factors like the intensity and duration of the original stimulus (Bonfoco et al. 1995, Syntichaki and Tavernarakis 2003, Benn and Woolf 2004). The mitochondria are believed to play an important role in the decision making process. It has been proposed that opening of the mitochondrial PTP only in a few mitochondria would result in lysosomal degradation of the affected mitochondria by autophagy. However, when more mitochondria become involved, the process would culminate in

apoptosis, and when the PTP opens in virtually all mitochondria of the cell, the result would be profound depletion of ATP followed by necrosis (Lemasters et al. 1998).

2.1.3. Autophagic cell death

The term autophagy refers to a process where a proportion of the cellular cytoplasm is engulfed into a double-membrane vesicle, and the resulting autophagosome is subsequently fused with a lysosome to permit degradation of its contents by lysosomal hydrolytic enzymes (Yuan et al. 2003, Marino and Lopez-Otin 2004, Shintani and Klionsky 2004). Autophagy occurs as a means to resist starvation, as well as for degradation of dysfunctional organelles and other cytoplasmic components (Yuan et al. 2003, Marino and Lopez-Otin 2004, Shintani and Klionsky 2004). Additionally, extensive autophagy may lead to a special form of cell death, i.e. autophagic cell death.

The most prominent feature of autophagic cell death is the appearance of large autophagic vacuoles. When most of the cytoplasm has been destroyed by autophagy, there is induction of apoptotic markers and phagocytosis of the cell remnants as in classical apoptosis. However, the apoptotic phase of cell death is short and markedly delayed (Clarke 1990, Zakeri and Lockshin 2002). The induction of autophagy appears to be regulated by the phosphatidylinositol-3-kinases (PI3Ks) though other factors are also involved. In nutrient rich conditions, signaling involving class I PI3K and the Akt-kinase can activate mTor, which arrests autophagy. During starvation, this pathway is inhibited, whereas the activity of class III PI3K promotes membrane events associated with autophagosome formation (Marino and Lopez-Otin 2004, Shintani and Klionsky 2004). There are several examples of cell death involving autophagic events in the absence of any significant apoptotic features. Also, in some cases cell death is reduced in cultured cells when autophagy is inhibited (Assuncao Guimaraes and Linden 2004, Marino and Lopez-Otin 2004). However, there is still debate on whether autophagy produces a special form of cell death, or is merely a non-causative, or even survival-promoting, phenomenon occurring during cell death in some stressful conditions (Yuan et al. 2003, Edinger and Thompson 2004). Future research on autophagy will provide better tools to be used in the evaluation of the role of autophagy in cell death.

2.1.4. Glutamate excitotoxicity

The term excitotoxicity refers to toxicity caused by abnormally intense stimulation of receptors activated by excitatory neurotransmitters. Glutamate is the major mediator of excitatory neurotransmission in the brain, and therefore it is not surprising, that glutamate is also the most important inducer of excitotoxicity (Dingledine et al. 1999, Arundine and Tymianski 2003). Glutamate excitotoxicity frequently occurs following acute insults such as brain ischemia, trauma, or epileptic seizures, and it is believed to be involved also in several neurodegenerative disorders (Lipton and Rosenberg 1994). Glutamate excitotoxicity may take place when the level of extracellular glutamate is elevated due to increased release, or decreased uptake of the neurotransmitter. Cell death due to glutamate excitotoxicity can occur with both necrotic and apoptotic mechanisms (Bonfoco et al. 1995, Lankiewicz et al. 2000, Zipfel et al. 2000).

The cellular effects of glutamate are mediated by three different classes of ionotropic glutamate receptors, as well as by metabotropic glutamate receptors (mGluRs) (Siegel et al. 1999, Arundine and Tymianski 2003). In general, the ionotropic receptors have an agonist-binding site and the associated ion channel in the same macromolecular complex. The ionotropic α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and kainate classes of glutamate receptors are collectively called “non-NMDA” receptors due to their structural similarity and overlapping pharmacological properties. Binding of glutamate to the extracellular part of the receptor opens the receptor ion channel. However the ion fluxes are soon terminated by a conformational change occurring in the receptor (desensitization) (Dingledine et al. 1999, Siegel et al. 1999). At the normal resting potential, the N-methyl-D-aspartate (NMDA) class of ionotropic glutamate receptors do not efficiently respond to glutamate due to blockage of the receptor ion channel by a magnesium ion. When the plasma membrane is depolarized, for instance due to high activity of the non-NMDA receptors, the magnesium block of the NMDA receptors is removed, and the receptor becomes permeable to ions (Kandel et al. 1995, Dingledine et al. 1999, Siegel et al. 1999). The mGluRs are structurally and functionally distinct from the ionotropic receptors. The

mGluRs are G-protein coupled receptors, and they mediate their effect by affecting intracellular signaling molecules, such as phospholipase C or adenylyl cyclase, rather than by directly controlling the activity of ion channels (Siegel et al. 1999).

As described in the previous chapters, excessive increase in the level of intracellular calcium activates several downstream events, which contribute to both necrotic and apoptotic cell death. In glutamate excitotoxicity, the role of calcium is critical (Choi 1987). The post-synaptic signaling induced by glutamate receptor activation results in an increased intracellular calcium level via several different mechanisms. Activation of the non-NMDA receptors leads to influx of sodium causing depolarization. However, some non-NMDA receptors are also permeable to calcium (Tanaka et al. 2000, Arundine and Tymianski 2003). The glutamate-induced depolarization also leads to calcium influx due to opening of voltage-sensitive calcium channels (VSCC) in the plasma membrane (Leist and Nicotera 1999, Arundine and Tymianski 2003). Under depolarized conditions, glutamate activates also the NMDA receptors. These receptors cause further sodium influx, and are potent activators of calcium-dependent intracellular signaling due to their high calcium permeability (Kandel et al. 1995, Aarts and Tymianski 2003, Arundine and Tymianski 2003). The mGluRs are also able to increase the level of intracellular calcium by provoking mobilization of calcium from the endoplasmic reticulum (Siegel et al. 1999). The quantity of calcium influx alone does not seem to predict the extent of cell death. Also the route of calcium influx may determine whether the excitotoxicity-associated events take place. Calcium influx through the NMDA receptors is considered to be particularly important with respect to glutamate excitotoxicity, possibly due to the close association of the receptors with certain mediators of calcium-activated signaling, such as nNOS (Sattler et al. 1998, Arundine and Tymianski 2003, Duchen 2004). However, in some conditions also the AMPA receptors may play a predominant role.

2.1.5. Cell death in disorders of the nervous system

Neuronal cell death is a feature of several neuropathological conditions. After acute insults, such as stroke, traumatic brain injury, or epileptic seizures, there is immediate

and extensive loss of neurons followed by delayed cell death when cells are lost at a slower rate. Also several neurodegenerative disorders, such as AD, PD, ALS, or Huntington's disease, are characterized by neuronal loss, which generally occurs in a slow and progressive manner. Cell death occurring under these conditions, especially in stroke and neurodegenerative tauopathies, will be discussed in more detail below.

The two main types of strokes are ischemic stroke and hemorrhagic stroke. The majority of the strokes are ischemic resulting from a severe reduction in cerebral blood flow (Bramlett and Dietrich 2004). As the energy metabolism of the brain is almost exclusively dependent on oxidation of glucose, the shortage of oxygen and glucose during ischemia leads to rapid depletion of ATP stores. Neurons need a continuous supply of energy for instance to maintain the ionic gradients responsible for polarization of their plasma membrane. Once the neuron is depolarized, there is influx of calcium through the voltage-sensitive channels, and subsequent release of neurotransmitters, such as glutamate, at the synaptic region. Also the re-uptake of glutamate is impaired due to energy depletion, and thus the level of extracellular glutamate is increased (Dirnagl et al. 1999, Bramlett and Dietrich 2004). There is a prominent role for glutamate excitotoxicity in ischemia. This has been demonstrated by the protective effect of glutamate receptor antagonists in animal models of ischemia (Simon et al. 1984, Ozyurt et al. 1988, Buchan et al. 1991). The cell death during ischemia occurs by both necrosis and apoptosis. In animal models of ischemia, calpain inhibition efficiently prevents cell death (Bartus et al. 1994), pointing to an important role for necrosis-associated phenomena. Morphological analysis also indicates that necrosis indeed is the predominant mechanism of cell death after ischemia (Li et al. 1998). However, also indicators of apoptosis have been found in ischemia (e.g. translocation of Bax to the mitochondria, release of cytochrome c from the mitochondria, increased expression of death receptors and their ligands, caspase-3, -8, and -9 induction, and internucleosomal DNA fragmentation), and interfering with several mediators of apoptosis has reduced the size of the infarct (Ferrer and Planas 2003, Liou et al. 2003). Further, there is also morphological evidence indicative of autophagy induction in ischemia (Nitatori et al. 1995).

In chronic neurodegenerative disorders, the underlying cause, as well as the course of cell death, is very difficult to evaluate. The amount of mechanistic information that can be obtained with human postmortem brain material is limited, and the available material often represents the late stages of the disease being contaminated by a significant extent of secondary pathology. There is also a shortage of good experimental models. Although neurodegenerative disorders constitute a heterogeneous group of diseases, there are certain common features in their neuropathology. This suggests that there might also be similarities in the mechanisms of cell death. One of the most conspicuous features in the neuropathology of neurodegenerative disorders is the presence of large protein aggregates either inside the neuronal and/or glial cells, or in the extracellular space (Shastry 2003, Ross and Poirier 2004). The main protein components of some of these aggregates are presented in table 1. The aggregating proteins generally form long filamentous protein polymers often rich in β-sheet conformation, and the proteins also contain similar post-translational modifications (ubiquitinylation, phosphorylation, oxidation, proteolytic truncation etc.) (Ross and Poirier 2004). It is conceivable that these modifications might have a role in the aggregation process. The close association of protein aggregation and neuronal degeneration is supported by the fact that mutations in the main protein species of the aggregates are the most common causes of dominantly inherited rare forms of these degenerative disorders (Ross and Poirier 2004). However, there is still debate, whether the voluminous protein aggregates are actually the cause of cellular degeneration, or may even represent a protective structure sequestering the more hazardous but poorly discernible aggregation intermediates (Tran and Miller 1999, Ross and Poirier 2004). Whatever the case, research on the upstream events of protein aggregation might unravel events that are more closely linked to the cell death in neurodegenerative disorders than the presence of the hallmark aggregates *per se*. In addition to protein aggregation, oxidative stress, proteasomal dysfunction, excitotoxicity, and mitochondrial injury have been linked with several neurodegenerative disorders (Bossy-Wetzel et al. 2004).

Table 1. Protein aggregation in some neurodegenerative diseases

Disease	Characteristic pathology	Main protein components
AD	Neuritic plaques Neurofibrillary tangles	Aβ peptide Hyperphosphorylated tau
PD	Lewy bodies	α-Synuclein
ALS	Ubiquitinated inclusions, Bunina bodies and hyaline bodies	Superoxide dismutase 1?, neurofilaments?
Huntington's disease	Intranuclear inclusions and cytoplasmic aggregates	Huntingtin
Prion diseases	Spongiform degeneration, amyloid, other aggregates	Prion protein

Data from references: (Wood et al. 2003, Ross and Poirier 2004).

Tauopathies are a group of neurodegenerative disorders characterized by the presence of intracellular (neuronal and glial) aggregates consisting mainly of filamentous polymers of the microtubule-associated protein tau. The most common form of tau-aggregate is the neurofibrillary tangle (NFT) (Buee et al. 2000, Lee et al. 2001). Normally tau promotes microtubule assembly, stabilizes microtubules, and facilitates the interaction of microtubules with other proteins and cellular elements (Buee et al. 2000). The sequence of events leading to pathology-associated tau accumulation, filament assembly, and formation of inclusions is still poorly understood. One common finding in neurodegenerative tauopathies is that tau accumulates in an abnormally hyperphosphorylated form. Indeed alterations in the phosphorylation of tau seem to occur prior to, or at least concomitant with, aggregation and cellular degeneration (Braak et al. 1994). Several kinases (e.g. glycogen synthase kinase 3 and cdk5) have been incriminated in the disease-associated tau hyperphosphorylation (Yamaguchi et al. 1996, Lee et al. 2001). Hyperphosphorylation might detach tau from the microtubules, and it could also promote the self-assembly of tau (Buee et al. 2000, Alonso et al. 2001, Lee et al. 2001, Avila et al. 2004). Tau polymerization may additionally be promoted by other covalent modifications, and by aggregation-promoting intracellular molecules (Buee et al. 2000, Avila et al. 2004). A causative role for aberrant tau biology in neurodegeneration is suggested for instance by the fact that mutations in the tau gene

give rise to a clinically heterogenous group of disorders referred to as frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17). The disease-associated tau mutations have been suggested to promote tau pathology by reducing the binding of tau protein to microtubules, by promoting the assembly of tau into filaments, or by altering the relative amounts of different tau splice isoforms (Buee et al. 2000, Lee et al. 2001). However it should be noted, that there are also families having hereditary frontotemporal dementia with linkage to chromosome 17, displaying tau-negative inclusions and lacking mutations in the tau gene (van Swieten et al. 2004).

Although there are common features in the neuropathology of tauopathies, these disorders differ for instance in the tau phosphorylation patterns, accumulating tau splice isoforms, tau filament and inclusion morphologies, affected cell types and brain regions, as well as in the clinical manifestation (Buee et al. 2000, Lee et al. 2001, Avila et al. 2004). AD is the most common tauopathy as well as the most common form of dementia. In some rare cases AD is caused by dominantly inherited gene mutations. However, the etiology of sporadic AD is poorly understood. Two different types of protein aggregates are found in the brains of patients suffering from AD. In addition to the intracellular NFTs, there are extracellular amyloid plaques, which are aggregates composed mainly of β-amyloid, a peptide enzymatically released from a larger amyloid precursor protein (APP) (Odle 2003, Bossy-Wetzel et al. 2004, Mattson 2004). Both NFTs and plaques are related to the cognitive deficiency in AD. However, the presence of these mature protein aggregates relatively poorly correlates with neuropsychological tests of cognitive function. For instance, the density of synapses is far more closely associated with the cognitive symptoms (Terry et al. 1991). The fact that only a minor proportion of the cells is actually undergoing cell death at any given time in the brains of AD patients, makes it difficult to estimate the predominant mechanisms of cell death. The biochemical evidence, such as activation of caspase-proteases, point to the activation of apoptotic mechanisms in AD (Raina et al. 2003, Dickson 2004, Mattson 2004). However, there is a shortage of morphological evidence for completion of the apoptotic program (Stadelmann et al. 1998, Stadelmann et al. 1999, Raina et al. 2003). There is also evidence for elevated lysosomal activity and autophagy in AD, paralleling the findings in some other neurodegenerative disorders (Yuan et al. 2003, Nixon et al.

2005). Whether these changes in neurodegenerative disorders, such as AD, represent an effort to remove potentially hazardous intracellular protein aggregates, or significantly contribute to neuronal demise, remains unclear. Nonetheless, the neuronal cell loss in AD is likely to be promoted by certain features of the disease e.g. oxidative stress, impaired cellular energy metabolism, dysregulated calcium homeostasis, excitotoxicity, and inflammation (Danysz et al. 2000, McGeer and McGeer 2003, Mattson 2004).

2.2. The cyclin-dependent kinase 5 (cdk5)

Cdk5 (alternative names: neuronal cdc2-like kinase, tau protein kinase II, PSSALRE) is a proline-directed serine/threonine protein kinase originally discovered based on its homology with the $p34^{cdc2}$ (cdk1) kinase (Meyerson et al. 1992). Cdk5 is inactive unless bound to cdk5-specific activator proteins p35 or p39 (Lew et al. 1994, Tsai et al. 1994, Tang et al. 1995), which represents a common theme in cdk regulation. Generally the cyclin-dependent kinases are involved in regulation of cell cycle progression. However the predominant expression of cdk5, and more strikingly of the cdk5 activator proteins, in post-mitotic neurons immediately led to the suggestion that this kinase possesses functions distinct from cell cycle regulation (Hellmich et al. 1992, Tsai et al. 1994, Tang et al. 1995). Indeed, cdk5 has been claimed to have a role in the development of the nervous system, in learning and memory, in synaptic signaling, and in regulation of cell biological events with special importance in the neuronal cells. Functional alterations in cdk5 have been suggested to contribute to neuronal cell death in neuropathological conditions. Although neurons are the predominant cell type with cdk5 activity, also non-neuronal functions for cdk5 have been proposed. The following review, however, will concentrate on the biology of cdk5 in the neuronal environment.

2.2.1. Structure of cdk5 and its activator proteins

The human cdk5, p35, and p39 genes are transcribed to single mRNA species encoding for proteins of 291, 307 and 367 amino acids, respectively. The primary structure of cdk5 protein is highly homologous to some other cyclin-dependent kinases, being 57 %

and 60 % identical, respectively, with cdc2 and cdk2 (Meyerson et al. 1992). The p35 and p39 proteins are 57 % identical (Tang et al. 1995). They do not appear to have significant sequence homology with other proteins, apart from the marginal homology with the cyclin box employed by various cyclins to interact with their cdk partners (Lew et al. 1994, Tsai et al. 1994, Tang et al. 1995). Nevertheless, the overall tertiary structure of the large C-terminal globular domain of p35 resembles that of cyclins (Tarricone et al. 2001). Both p35 and p39 contain N-terminal myristoylation signals, and indeed both proteins are preferentially found attached to membranes (Patrick et al. 1999, Patzke and Tsai 2002). Also, the N-terminal parts of p35 and p39 are likely less tightly structured, and may act in protein-protein interactions (Tarricone et al. 2001). A large number of proteins do interact with p35 and/or p39, and it has been suggested that the activator proteins might also function as adaptors, targeting cdk5 to large molecular complexes, and to the vicinity of its substrates (Lim et al. 2003).

2.2.2. Regulation of cdk5

Regulation of cdk5 takes place at several different levels. There are significant alterations in the protein expression of cdk5 and its activator proteins during neuronal differentiation and development of the nervous system, as well as in conditions associated with neuronal growth and plasticity. Post-translational mechanisms, such as phosphorylation of cdk5, and proteolytic degradation and truncation of cdk5 activators, provide more rapid mechanisms for the regulation of cdk5. The role of p35 activator protein in cdk5 regulation has been much more extensively studied than that of p39. Although there seems to be similarities in the post-translational mechanisms employed in the regulation of p35 and p39, there are evident differences e.g. in their temporal and spatial expression patterns.

Cdk5, p35, and p39 protein expression. The protein expression levels of cdk5, p35, and p39 are markedly altered during the course of development of the central nervous system. Studies performed on rodents have revealed that especially the cdk5 activator proteins are highly neuron-specific, and they are first detected when the neurons become post-mitotic (Tsai et al. 1993, Tsai et al. 1994, Zheng et al. 1998). The level of

cdk5 progressively increases during the development of the nervous system, and maximum expression is detected in the adult brain (Kobayashi et al. 1993, Tsai et al. 1993). Expression of cdk5 is observed throughout the brain with the highest levels being found in the hippocampus and mesencephalon (Delalle et al. 1997). Also the p35 level increases during the course of brain development, peaking at the time of birth. However, p35 is downregulated soon after birth, although p35 expression remains high in some specific brain regions, such as the hippocampus (Uchida et al. 1994, Tomizawa et al. 1996). The expression of p39 differs temporally and spatially from that of p35, being low during early development. However, between one to three weeks after birth, p39 is highly expressed throughout the brain, whereas in mature brains, p39 expression is relatively restricted to the cerebellum (Cai et al. 1997). Subcellularly cdk5 is localized to cell bodies in the developing nervous system, whereas in adult rodents, cdk5 is predominantly present in the axons, and detectable also in nuclei (Tsai et al. 1993, Ino and Chiba 1996, Matsushita et al. 1996, Tomizawa et al. 1996). In cultured cerebellar macroneurons cdk5, is especially concentrated at the distal tips of growing axons (Pigino et al. 1997). However, p35 mainly locates to the cell bodies and dendrites, and axonal p35 is only detectable during the development and soon after birth (Matsushita et al. 1996, Tomizawa et al. 1996). Also p39 may be more abundant in the neuronal somata and dendrites than in axons of the adult rat brain (Honjyo et al. 1999, Jeong et al. 2003). Nevertheless, in cultured neuronal cells at least p35 is also present in the growth cones of axons (Paglini et al. 1998).

The presence of cdk5 activator proteins only in post-mitotic neurons suggests that neuronal differentiation could activate the expression of these proteins. This is supported by upregulation of cdk5, p35, and p39 in cultured cell lines when neuronal differentiation is induced by retinoic acid (Fu et al. 2002, Haque et al. 2004, Lee and Kim 2004). Also neurotrophic factors stimulate p35 expression in cultured neurons and PC12 cells (Tokuoka et al. 2000, Harada et al. 2001). Further, when neurons are cultured on laminin, an extracellular matrix protein capable of stimulating axonal extension, p35 protein expression, but not p39 expression, is induced. This is associated with redistribution of p35 and cdk5 to the axonal growth cones (Pigino et al. 1997, Paglini et al. 1998). Additionally, increased neuronal activity and plasticity *in vivo* have

been observed to be associated with upregulation of cdk5 or p35 proteins, e.g. this has been detected following chronic electroconvulsive seizures (Chen et al. 2000), during kindling progression (Tomizawa et al. 2000), axonal regeneration (Namgung et al. 2004), associative learning (Fischer et al. 2002), and in conjugation with the adaptive changes following chronic cocaine exposure (Bibb et al. 2001a). Changes in the protein levels of cdk5 and/or its activators have been also observed during the course of neuronal cell death and in various neuropathological conditions, as will be discussed later.

Degradation of cdk5 activator proteins. A common feature in the regulation of cyclin-dependent kinases is a controlled degradation of the regulatory cyclin proteins by the ubiquitin-proteasome pathway at a certain stage of the cell cycle. Although cdk5 mainly functions in non-dividing cells, a similar molecular mechanism may be employed in the regulation of cdk5. p35 has been observed to be a short-lived protein, which is degraded after its ubiquitination by the ubiquitin-proteasome pathway. Phosphorylation of p35 by cdk5 appears to favor p35 degradation (Patrick et al. 1998, Saito et al. 2003). The cdk5-induced degradation of p35 might have an autoregulatory role by providing a means for negative feedback regulation of cdk5 activity. Also, the level of p35 phosphorylation, and the rate of its proteasomal degradation, is higher in fetal rat brain tissue than in adult tissue, findings which suggest that p35 degradation could be developmentally regulated (Saito et al. 2003).

Calpain-mediated cleavage of the cdk5 activator proteins. The cdk5 activator proteins p35 and p39 have been found to be cleaved to smaller p25 and p29 fragments, respectively, by the calcium-activated calpain-protease (Kusakawa et al. 2000, Lee et al. 2000, Patzke and Tsai 2002). In accordance with the observed activation of calpain during cell death, cleavage of the cdk5 activator proteins is often detected in neurotoxic conditions *in vitro* and *in vivo*, especially when a prominent calcium influx is known to be involved (Kusakawa et al. 2000, Lee et al. 2000, Nath et al. 2000, Patzke and Tsai 2002). However, activation of calpain is likely not to occur exclusively under conditions involving cell death. Calpain has been implicated in remodeling of cytoskeleton-membrane attachments, in intracellular signal transduction, and in the cell cycle (Goll et

al. 2003). Calpain also seems to be participate in long-term potentiation (LTP) (Goll et al. 2003), an experimental model of learning and memory involving calcium-dependent enhancement of the post-synaptic responses following intense electrical stimulation. Indeed, the post-synaptic signaling evoked by the neurotransmitter glutamate induces calpain-mediated cleavage of the cdk5 activator proteins during excitotoxic conditions (Lee et al. 2000, Patzke and Tsai 2002). However, the occurrence and possible role of glutamate-induced cleavage of cdk5 activators in physiological conditions remains to be demonstrated. Also the properties of the substrate appear to affect the rate of the calpain-mediated cleavage. Cdk5-induced phosphorylation of p35 reduces calpain-mediated p35-cleavage, again suggesting possible autoregulation. Accordingly, p35 cleavage is suppressed in fetal brain tissue where the level of p35 phosphorylation is high (Saito et al. 2003).

The calpain-mediated cleavage of the cdk5 activator proteins has several types of effects on the function of cdk5. The p25 fragment of p35 contains the globular domain responsible for cdk5 binding and activation. However, it has lost the less ordered N-terminal part of p35, which harbors the myristoylation site, and which may mediate the interactions between p35 and other proteins (Poon et al. 1997, Patrick et al. 1999, Tarricone et al. 2001, Amin et al. 2002). Due to the loss of myristoylation, both p25 and p29 are preferentially located in the cytosol, in contrast to p35 and p39, which are predominantly membrane bound (Patrick et al. 1999, Kusakawa et al. 2000, Lee et al. 2000, Patzke and Tsai 2002). Thus the cleavage of the activator proteins results in a qualitative change in cdk5 function, and the kinase is allowed access to an alternative subset of substrates. Calpain also provokes a quantitative change in cdk5 activity, as p25 activates cdk5 more effectively than p35 (Patrick et al. 1999, Amin et al. 2002). Further, the p25 and p29 proteins have markedly increased half-lives when compared to the uncleaved proteins (Patrick et al. 1999, Patzke and Tsai 2002). It is possible that the calpain-cleaved cdk5 activator proteins can escape from the phosphorylation-induced proteasomal degradation which has been proposed as a mechanism of negative feedback regulation (Patrick et al. 1998). This could lead to constitutive kinase activity.

Regulation of cdk5 activity by phosphorylation. The availability of cyclins is a major determinant of cdk activity. However, also phosphorylation is widely employed in the regulation of cdks. In this respect, cdk5 possesses some general features of cdk regulation, however there are also significant differences. In contrast to the other cdks, cdk5 is highly active also in the absence of phosphorylation (Qi et al. 1995). There is some controversy on whether phosphorylation of cdk5 by the cdk-activating kinase (CAK) on Ser159 at the activating T-loop (corresponding to Thr160 in cdk2 and Thr161 in cdc2) actually alters cdk5 activity. CAK has been reported either to induce cdk5 activity *in vitro*, or to have no effect (Poon et al. 1997, Rosales et al. 2003). Also casein kinase I (CKI) has been observed to phosphorylate Ser159 and to stimulate cdk5 activity (Sharma et al. 1999b). Phosphorylation of cdk5/p35 by CKI has been implicated in the transient activation of cdk5 induced by a mGluR agonist in striatal slice cultures, although the phosphorylation site in this context has remained undetermined (Liu et al. 2001). At least two different kinases phosphorylate cdk5 on Tyr15. In contrast to other cdks, phosphorylation at Tyr15 enhances cdk5 activity. c-Abl, a kinase involved in the development of the nervous system, phosphorylates Tyr15 of cdk5, and this activity is enhanced by the putative adaptor protein Cables (Zukerberg et al. 2000). Phosphorylation of Tyr15 of cdk5 by the fyn kinase is involved in the signaling activated by semaphorin-3A, a guidance cue for axons and dendrites (Sasaki et al. 2002). Also signaling induced by ephrin-A5, a repulsive axonal guidance cue, evokes phosphorylation of cdk5 at Tyr15 by an unidentified kinase (Cheng et al. 2003). Phosphorylation of cdk5 at Thr14 by an unidentified kinase purified from the bovine thymus has been observed to inhibit cdk5 activity (Matsuura and Wang 1996). Nevertheless, phosphorylation of Thr14 has not been demonstrated *in vivo*. Phosphorylation of p35 does not seem to directly affect its ability to activate cdk5 (Saito et al. 2003).

Regulation of cdk5 activity by binding of other proteins. Several inhibitory proteins regulate the activity of cell cycle-associated cdks (Obaya and Sedivy 2002). However, cdk5 is not affected by the inhibitors modulating the activity of the other cdks (Lee et al. 1996). Nevertheless, there are some reports suggesting that also cdk5 activity might be affected by binding of proteins other than p35 or p39, although this concept is not well

established. The present evidence suggests that proteins such as the ribosomal protein L34, DNA binding protein dbpA, casein kinase 2, and a previously uncharacterized C42 protein possess the ability to bind to cdk5 or p35, and inhibit cdk5 activity (Moorthamer and Chaudhuri 1999, Moorthamer et al. 1999, Ching et al. 2002, Lim et al. 2004). Additionally, a nuclear protein SET appears to activate cdk5/p35 (Qu et al. 2002). The molecular mechanisms employed in the regulation of cdk5 are summarized in figure 1.

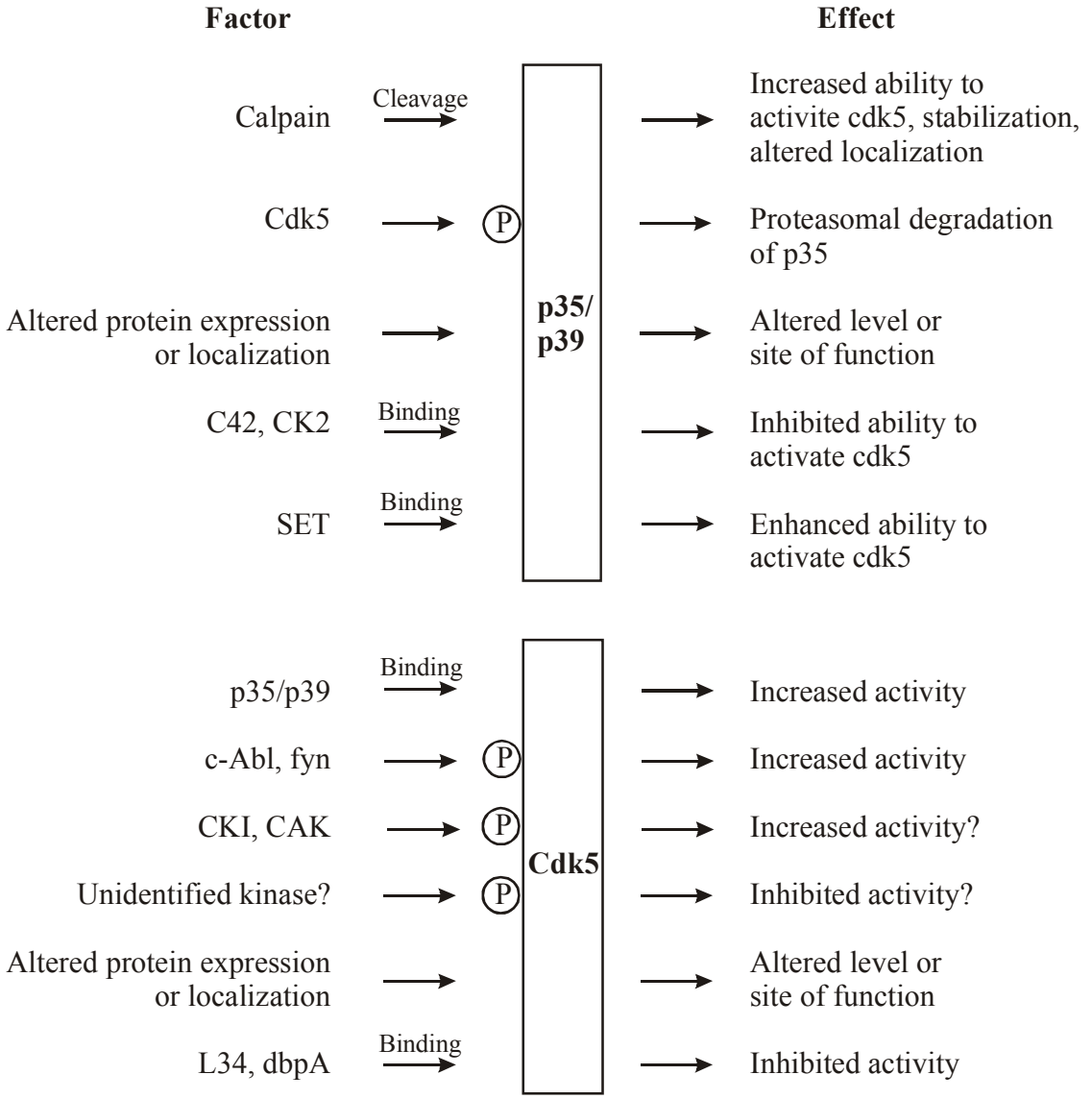

Figure 1. Summary of the molecular mechanisms employed in the regulation of cdk5. The figure shows the factors affecting p35/p39 and cdk5, and the proposed effects of these factors on protein function.

2.2.3. Biological functions of cdk5

A large number of cdk5 substrates are known. In general, the cell biological functions of the cdk5 substrates are most often related to the cytoskeleton, synaptic vesicle trafficking, cellular adhesion, or to intracellular signal transduction. Via its involvement in these cell biological processes, cdk5 is able to participate in fundamental neurobiological events ranging from the development of the neuronal architecture to synaptic transmission.

Neuronal migration and process outgrowth. Studies performed on mice lacking cdk5, p35, or p39 have established an important role for cdk5 in the proper migration of neurons during the development of the nervous system. Mice lacking cdk5 suffer from developmental abnormalities related to defective neuronal migration in many brain areas including the cerebral cortex, hippocampus and cerebellum (Ohshima et al. 1996, Gilmore et al. 1998). In mice lacking p35, the defect in neuronal migration is more restricted involving mainly the cerebral cortex (Chae et al. 1997), whereas in mice deficient in p39 there are no obvious abnormalities (Ko et al. 2001). The target proteins and molecular mechanisms involved in the regulation of neuronal migration by cdk5 are still inadequately known. Cdk5 could affect migration by regulating the cytoskeleton, cellular adhesion, or the response to guidance cues (Gupta and Tsai 2003).

Several studies suggest that cdk5 is needed for proper outgrowth of neuronal processes since prevention of the function of cdk5 significantly reduces the extent of process outgrowth in cultured neurons (Nikolic et al. 1996, Pigino et al. 1997). The response to some external growth promoting signals may be mediated through induced expression of p35 or p39 (Xiong et al. 1997, Paglini et al. 1998, Harada et al. 2001, Desbarats et al. 2003). In addition, cdk5 is believed to be involved in the regulation of the direction of neuritic growth at the growth cones. There is evidence for defective axonal guidance in mice lacking p35 (Kwon et al. 1999), and the cellular responses to some guidance cues seem to be mediated in a cdk5-dependent manner (Ledda et al. 2002, Sasaki et al. 2002, Cheng et al. 2003).

Learning and memory. In mice exposed to stressful conditions, the expression and activity cdk5 are induced in the fibers of septohippocampal cholinergic neurons. The cdk5 inhibitor butyrolactone I prevents associative learning related to context-dependent fear in these conditions (Fischer et al. 2002). Furthermore, mice overexpressing p25 at low level display improved performance in some tasks measuring learning and memory (Angelo et al. 2003). Cdk5 phosphorylates several substrates that could mediate an effect on learning and memory. The LTP-reducing effect of the cdk5 inhibitor roscovitine is thought to result from decreased NMDA-evoked currents when cdk5-mediated phosphorylation of the NR2A subunit of the NMDA receptor is prevented (Li et al. 2001).

Regulation of the cytoskeleton. At the molecular level, many of the neurobiological actions of cdk5 are likely to be at least partly mediated through modulation of the neuronal cytoskeleton. In neurons, cdk5 has been shown to regulate all of the main cytoskeletal elements (microtubules, actin filaments, intermediate filaments) either by directly phosphorylating the structural components of the cytoskeleton, or by phosphorylating proteins regulating cytoskeletal dynamics.

Cdk5 regulates microtubule dynamics in part via phosphorylation of microtubule-associated proteins (MAPs). In general, the MAPs bind to microtubules, promote their assembly, stabilize them, and organize them into bundles. Tau is a MAP which is especially concentrated in axons. Cdk5 has been observed to bind to tau, and to phosphorylate it at several different sites (Ishiguro et al. 1992, Baumann et al. 1993, Sobue et al. 2000, Lund et al. 2001). In a cell-free system the cdk5-mediated phosphorylation of tau reduced the ability of tau to associate with microtubules, and prevented tau-induced promotion of microtubule polymerization (Wada et al. 1998). Additionally, suppression of cdk5 or p35 expression has been shown to decrease axonal elongation as well as to reduce the phosphorylation of MAP1b, another MAP, in the distal ends of growing axons of cerebellar macroneurons (Pigino et al. 1997, Paglini et al. 1998). Regulation of the microtubule cytoskeleton may be important with respect to the effect of cdk5 on neuronal migration. The proteins doublecortin, NUDEL, and focal adhesion kinase (FAK) have been suggested as cdk5 target candidates, that might

mediate the effect of cdk5 on neuronal migration in a microtubule-dependent manner (Niethammer et al. 2000, Sasaki et al. 2000, Xie et al. 2003, Tanaka et al. 2004).

In neurons, an active site of actin filament assembly and disassembly are the growth cones, which are critical for elongation of the neurites. Interestingly, cdk5 promotes neurite outgrowth, and the cdk5, p35, and p39 proteins are concentrated at the edges of neuronal growth cones in the vicinity of actin filaments (Nikolic et al. 1996, Humbert et al. 2000). The best studied actin-associated target of cdk5 is the Pak1 kinase (Nikolic et al. 1998, Rashid et al. 2001), a Rac1 effector, which is involved in neurite outgrowth and growth cone collapse response (Nikolic 2002).

In differentiated neurons, the main type of intermediate filaments are the neurofilaments (NFs), which are dynamic components of the axonal cytoskeleton. NFs are involved in the maintenance of the axonal caliber. NF-H and NF-M contain C-terminal KSP-repeat motifs that are extensively phosphorylated *in vivo*, and which are targets for phosphorylation by cdk5 (Sun et al. 1996, Sharma et al. 1999a). Phosphorylation of NFs by cdk5 alters the intracellular distribution of NFs, and reduces the rate of axonal transport of these proteins (Ackerley et al. 2003, Shea et al. 2004).

Regulation of cellular adhesion. N-cadherin is an adhesion molecule having crucial roles during the development of the nervous system. In general, cadherin molecules form homophilic contacts with cadherins on adjacent cells, whereas the intracellular anchor proteins, catenins, connect these adhesion sites to the intracellular actin-cytoskeleton. Cdk5-p35 has been observed to associate with the β-catenin–N-cadherin complex, and to phosphorylate β-catenin (Kwon et al. 2000, Kesavapany et al. 2001). Cdk5 activity decreases β-catenin–N-cadherin interaction and reduces cellular adhesion (Kwon et al. 2000). Decreased cdk5 activity evoked by depolarization of hippocampal neurons leads to redistribution of β-catenin from the dendritic shafts to the spines, an effect which is mimicked by the cdk5 inhibitor roscovitine (Schuman and Murase 2003).

Regulation of synaptic vesicle release and endocytosis. Cdk5 has been suggested to modulate several steps of synaptic vesicle trafficking in the presynaptic terminals. Cdk5 phosphorylates the P/Q type VSCCs, and thereby inhibits calcium influx into the synaptic terminals following depolarization. This reduces the subsequent exocytosis of neurotransmitter-containing vesicles (Tomizawa et al. 2002). Cdk5 also binds to and phosphorylates Munc-18, which reduces the affinity of Munc-18 for syntaxin 1A, a plasma membrane receptor for synaptic vesicles. This has been suggested to allow syntaxin 1A to interact with proteins on the synaptic vesicles, and to promote neurotransmitter release (Shuang et al. 1998, Fletcher et al. 1999). Thus cdk5 seems to inhibit an early step in the synaptic vesicle release, and to augment a later step in the process. Synaptic vesicle endocytosis is triggered following dephosphorylation of key proteins by the calcium-dependent protein phosphatase, calcineurin. Cdk5 has been suggested to participate in the rephosphorylation of some of these proteins (amphiphysin I, dynamin I, synaptojanin 1) (Floyd et al. 2001, Tan et al. 2003, Tomizawa et al. 2003, Lee et al. 2004).

Post-synaptic signaling. Binding of dopamine to its receptors results either in stimulation or inhibition of cAMP production depending on which dopamine receptor type has been activated. A major downstream effector of cAMP is the protein kinase A (PKA). In addition to other substrates, PKA phosphorylates the protein phosphatase-1 (PP-1) inhibitors DARPP-32 (dopamine and cAMP-regulated phosphoprotein, 32 kDa) and PP inhibitor-1. This leads to reduced PP-1 activity, and thereby to increased phosphorylation of other PKA substrates (Hemmings et al. 1984, Endo et al. 1996). In the striatum, cdk5 modulates dopamine signaling by negatively regulating the effects of PKA. Under basal conditions, cdk5 phosphorylates DARPP-32, and converts it into an inhibitor of PKA (Bibb et al. 1999). Additionally, cdk5 phosphorylates the PP inhibitor-1, making it a poor substrate for PKA (Bibb et al. 2001b). When dopamine D1 receptors are activated, the cdk5 phosphorylation site of DARPP-32 becomes dephosphorylated by PP-2A, which abolishes the inhibitory effect of DARPP-32 on PKA (Nishi et al. 2000).

Glutamate-induced cdk5 activation has been suggested to provoke feedback effects on post-synaptic glutamate signaling. Cdk5 phosphorylates the NR2A subunit of the NMDA class of glutamate receptors (Li et al. 2001). This appears to amplify NMDA-mediated signaling, since inhibition of cdk5 activity by roscovitine can reduce NMDA evoked currents and LTP in hippocampal CA1 neurons (Li et al. 2001). Calpain activation might augment the cdk5-mediated effect on the NMDA receptor, as Cdk5-p25 is more efficient than cdk5-p35 in inducing NR2A phosphorylation and NMDA evoked currents in transfected HEK293 cells (Wang et al. 2003). Cdk5 also phosphorylates PSD-95, a scaffolding protein that links the NMDA receptors to the cytoskeleton and to signaling molecules at the post-synaptic density. The cdk5-mediated phosphorylation reduces the self-association of PSD-95. This has been suggested to negatively regulate the synaptic clustering of its target proteins, such as NMDA receptors (Morabito et al. 2004).

Stimulation of group I mGluRs in neostriatal slice cultures activates a signaling pathway involving inositol 1,4,5-triphosphate (IP_3)-induced release of calcium from the endoplasmic reticulum. This causes calcineurin-mediated induction of CKI, and finally transient phosphorylation and activation of cdk5-p35 (Liu et al. 2002). Under these conditions, stimulation of mGluRs enhances cdk5-mediated DARPP-32 phosphorylation as well as calcium influx through VSCCs in a manner dependent on cdk5 and DARPP-32 (Liu et al. 2001).

2.2.4. Cdk5 in neuronal cell death and in neuropathological conditions

Cdk5 has important functions throughout the life of neurons, and it may have a role during the death of these cells as well. When neurons encounter stressful conditions, there are significant changes in cdk5. With respect to human pathological conditions, elevated levels of cdk5 or its activator proteins are often detected in affected neurons. Also calpain-mediated cleavage of cdk5 activators, which produces increased activity and functional alterations in cdk5, has been suggested to occurr in several neuropathological conditions. There is an accumulating body of evidence suggesting that abnormally intense activation of cdk5 would promote neuronal death. Nevertheless,

some studies imply that induction of cdk5 could also support neuronal survival. It is conceivable that the preferential effect of cdk5 in stressful conditions might depend on factors like intensity, duration, and subcellular location of cdk5 activation, as well as on neuronal cell type, and on other factors determining the internal state of the cell.

Alterations in cdk5 during neuronal death and in neuropathological conditions. Studies regarding the regulation of cdk5 in different experimental models of neuronal death have revealed a frequent occurrence of calpain-mediated cleavage of cdk5 activators. This is consistent with the common involvement of elevated intracellular calcium levels in neuronal death. Calpain-mediated cleavage of p35 or p39 has been reported in neuronal cell cultures exposed to calcium ionophores, maitotoxin, glutamic acid, β-amyloid, H_2O_2, colchicine, or staurosporine (Kusakawa et al. 2000, Lee et al. 2000, Nath et al. 2000, Patzke and Tsai 2002, Canudas et al. 2004). *In vivo* cleavage of p35 has been detected in malonate-injured rat brains (Nath et al. 2000). Also the protein levels of cdk5 and p35 are altered during the process of neuronal cell death. However, there is evidence for both elevated (Canudas et al. 2004, Zhang et al. 2004a) and reduced (Shirvan et al. 1998, Vartiainen et al. 2002) cdk5 and p35 levels in neuronal cultures subjected to toxic conditions. *In vivo* accumulation of cdk5 and p35 has been observed in cell bodies and nuclei during the late stages of apoptotic neuronal death (Henchcliffe and Burke 1997, Zhang et al. 1997, Neystat et al. 2001), although this might be partially explained by an altered localization of these proteins.

Changes in the regulation of cdk5 take place in several human neuropathological conditions, and in experimental models mimicking these disorders. Currently, there are no reports investigating cdk5 in human cases affected by ischemic stroke. However, in rat brains, elevated cytoplasmic and nuclear immunoreactivities for p35 and cdk5 are detected transiently 1 – 3 h after occlusion of the middle cerebral artery (Hayashi et al. 1999). Ischemia also causes calpain-mediated cleavage of both p35 and p39 in experimental animals (Lee et al. 2000, Nath et al. 2000, Patzke and Tsai 2002).

In AD there is accumulation of cdk5 in neurons harboring NFTs (Yamaguchi et al. 1996). However, the highest cdk5 levels are detected in neurons displaying lower

immunoreactivity for phosphorylated tau and no staining for ubiquitin, which suggests preferential expression of cdk5 in neurons with early neurofibrillary degeneration (Pei et al. 1998). In transgenic mice mimicking the amyloid pathology of AD, the accumulation of cdk5 is a rare event and confined to dystrophic neurites (Tomidokoro et al. 2001). Detection of calpain-mediated cleavage of cdk5 activators in human brain tissue is markedly hampered by the extensive proteolysis of these proteins during the postmortem period (Taniguchi et al. 2001). Thus, it is not surprising that there are highly conflicting results on the p25 protein levels in brains of patients with AD (Patrick et al. 1999, Takashima et al. 2001, Taniguchi et al. 2001, Yoo and Lubec 2001, Tseng et al. 2002, Tandon et al. 2003). However, in AD there is increased immunolabeling of NFT-containing neurons with an antibody recognizing the C-terminus of p35. As immunoreactivity for the N-terminus of p35 is not elevated in NFTs, this suggests that it represents the accumulation of the p25 proportion of p35 (Patrick et al. 1999). In transgenic mice mimicking the amyloid pathology of AD, both increased and unaltered levels of p25 have been reported (Otth et al. 2002, Tandon et al. 2003).

Cdk5 expression is also increased in the motor neurons of patients with ALS. Some, but not all, neurons containing accumulated NFs characteristic for ALS also display augmented cdk5 immunoreactivity (Nakamura et al. 1997b, Bajaj et al. 1998). Additionally, the ALS-mimicking pathology in mice overexpressing mutated superoxide dismutase 1 (SOD1) is associated with accumulation of cdk5 in the spinal motor neurons. Also increased cleavage of p35 to p25, and elevated cdk5 activity is detected in the spinal cord of these animals (Nguyen et al. 2001). Furthermore, cdk5 and p35 are found in Lewy bodies of PD and diffuse Lewy body disease (Brion and Couck 1995, Nakamura et al. 1997a). In mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to mimic the pathological alterations of PD, the expression of cdk5 is increased in the dopaminergic neurons of the substantia nigra, and also elevated cdk5 activity and calpain-mediated cleavage of p35 are detected (Smith et al. 2003). Additionally, calpain-mediated cleavage of p35 has been reported in rats with experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis (Schneider et al. 2004).

In summary, altered protein expression of p35-cdk5 and/or calpain-mediated cleavage of cdk5 activator proteins frequently co-occurs with neuronal death and protein accumulation. However, based on these findings alone, it cannot be concluded whether the changes in cdk5 contribute to or are a consequence of these processes.

The role of cdk5 in neuronal death. Several pieces of evidence do suggest that the changes in the function of cdk5 in stressful conditions promote the process of neuronal cell death. In neuronal cultures, cdk5 inhibitors reduce cell death induced by a wide range of neurotoxic insults, such as exposure to β-amyloid, colchicine, the glutathione synthase inhibitor buthionine sulfoxamine, or staurosporine, as well as by withdrawal of potassium and serum, or lack of neurotrophic factors (Alvarez et al. 1999, Lee et al. 2000, Jorda et al. 2003, Weishaupt et al. 2003). Also, more specific inhibition of cdk5 by an antisense oligonucleotide approach, or by overexpression of a polypeptide inhibitor of cdk5 (cdk5 inhibitory peptide, CIP), reduces β-amyloid-induced upregulation of apoptotic markers and neuronal death (Alvarez et al. 1999, Zheng et al. 2005). Moreover, overexpression of a dominant-negative mutant form of cdk5 decreases glutamate-induced apoptotic changes (Gong et al. 2003). In an experimental model of ischemia, cdk5 has been shown to aggravate neuronal cell loss, as inhibition of cdk5 by virus-mediated overexpression of dominant-negative cdk5 significantly reduces ischemia-induced neuronal death in the hippocampus (Wang et al. 2003). There is also evidence suggesting that inhibition of cdk5 can reduce neuronal degeneration in experimental animal models of neurodegenerative disorders. Expression of dominant-negative cdk5 reduces MPTP-induced loss of dopaminergic neurons (Smith et al. 2003). Furthermore, cdk5 inhibitors reduce NF and tau phosphorylation as well as loss of Purkinje neurons in an animal model of Niemann-Pick type C disease (Zhang et al. 2004b), a neurodegenerative lysosomal storage disorder. The pathology in these animals involves activation of cdk5 (Bu et al. 2002).

Pronounced calpain-mediated cleavage of cdk5 activator proteins has been commonly suggested to deregulate cdk5, as calpain evokes increased and prolonged cdk5 activity, and releases the active enzyme to the cytosol. Indeed, overexpression of p25 in a

neuronal cell line has induced cell death (Hamdane et al. 2003), whereas in neuronal cultures, p25 overexpression results in a greater number of condensed nuclei when compared to p35 overexpression (Patrick et al. 1999). However, it should not be overlooked, that cdk5 can promote neuronal death *in vitro* also independently of calpain-mediated cleavage of cdk5 activators (Weishaupt et al. 2003).

Several downstream mechanisms have been proposed to mediate the neuronal death-promoting effects of cdk5. Cdk5 might augment neuronal loss by modulating signaling systems ubiquitously associated with cell death. Neuronal characteristics do not seem to be required for cdk5-mediated cell death, as overexpression of cdk5 and p25 promotes apoptotic events in a non-neuronal cell line (Patrick et al. 1999). Indeed, cdk5 has been shown to phosphorylate p53, a transcription factor regulating cell cycle progression and apoptosis, leading to increased p53 levels and to stimulation of p53-responsive genes, such as Bax (Zhang et al. 2002). Cdk5 also downregulates signaling related to extracellular signal-regulated kinases 1 and 2 (ERK1/2), which could promote cell death, as activation of ERK1/2 has been suggested to trigger defense responses following neuronal injury (Hetman and Gozdz 2004). Cdk5 phosphorylates and thereby inhibits the mitogen-activated protein kinase kinase 1 (MEK1), which reduces the phosphorylation and the activity of its target ERK1/2 (Sharma et al. 2002). Additionally, cdk5 phosphorylates the Ras guanine nucleotide releasing factor 2 (RasGRF2), which results in decreased Rac activity, and subsequently reduced ERK1/2 activity through a still uncharacterized mechanism (Kesavapany et al. 2004). Moreover, cdk5 phosphorylates and inhibits the activity of the prosurvival transcription factor MEF2 (myocyte enhancer factor 2) (Gong et al. 2003). Overexpression of MEF2 which is resistant to cdk5-mediated phosphorylation, reduces neuronal cell death induced by cdk5-p25 overexpression, H_2O_2, or glutamate. Some of the effects of cdk5 on cell death are mediated by factors that are unique to neuronal cells. In ischemia, there is AMPA receptor-evoked activation of cdk5, and subsequent cdk5-mediated phosphorylation of the NMDA receptors, which augments NMDA currents and neuronal loss (Wang et al. 2003). This finding implicates cdk5 in the basic mechanisms of glutamate excitotoxicity. The fact that cdk5-specific inhibitors block mitochondrial dysfunction

induced by toxic treatments suggests that cdk5 affects relatively early events of cell death (Weishaupt et al. 2003).

When regarding the chronic neurodegenerative disorders, cdk5 activation has been suggested to affect neuronal degeneration in particular by promoting cytoskeletal dysfunction and/or abnormal accumulation of cytoskeletal proteins. The cdk5-induced phosphorylation of tau has been frequently proposed to have pathological consequences. However, the cdk5-mediated phosphorylation of NFs has been hypothesized even to be beneficial by preventing more hazardous cdk5-involving phosphorylation events (Nguyen et al. 2001). As mentioned earlier, cdk5 and p35 are found in various pathology-associated neuronal inclusions containing abnormally hyperphosphorylated tau or NF proteins. Also calpain-mediated cleavage of cdk5 activator proteins has been proposed to occur in some neurodegenerative disorders, although detection of this phenomenon in human brain material is hampered by postmortem changes. Moreover, cdk5 is able to phosphorylate both tau and NF proteins (Ishiguro et al. 1992, Baumann et al. 1993, Sun et al. 1996, Sharma et al. 1999a, Lund et al. 2001). In particular, calpain-mediated cleavage of p35 has been claimed to affect the potential of cdk5 to promote hyperphosphorylation of tau. *In vitro* and in transfected cell cultures, p25 appears to be more potent than p35 in promoting cdk5-induced tau phosphorylation (Patrick et al. 1999, Hashiguchi et al. 2002). Transgenic mice overexpressing p25 have shown alterations in the phosphorylation of tau and NF proteins, although the extent of these changes varies significantly (Ahlijanian et al. 2000, Takashima et al. 2001, Bian et al. 2002). The most prominent neuropathological effects have been observed in mice having postnatally induced overexpression of p25. These animals display neuronal loss, increased phosphorylation of NF and tau proteins as well as accumulation of insoluble and filamentous tau in association with NFT-like pathology (Cruz et al. 2003). Further, introduction of p25 significantly enhances the NFT-like pathology in mice overexpressing mutated human tau (Noble et al. 2003). Overexpression of p35 in mice results in redistribution of NF proteins, whereas no tau-involving changes are detected (Van den Haute et al. 2001). However, no actual comparison of the effects of p25 and p35 in transgenic mice has been reported. There is also some evidence suggesting that cdk5 might modulate the metabolism related to the formation of extracellular amyloid

deposits in AD as well. Cdk5 has been reported to phosphorylate APP (Iijima et al. 2000) and to modify its proteolytic processing (Liu et al. 2003, Ryder et al. 2003, Taru et al. 2004), whereas cdk5-mediated phosphorylation of the C-terminal fragment of presenilin-1 increases the stability of the protein (Lau et al. 2002).

The neuroprotective role of cdk5. Some evidence indicates that activation of cdk5 modulates neuronal signal transduction in a manner that promotes survival. Cdk5 has been observed to negatively regulate the c-Jun N-terminal kinase 3 (JNK3), which is a mitogen-activated protein kinase (MAPK) believed to promote neuronal cell death e.g. in the context of excitotoxicity (Yang et al. 1997). Cdk5 phosphorylates JNK3, leading to reduced JNK3 activity and c-Jun phosphorylation in response to ultraviolet irradiation (Li et al. 2002). In neuronal cultures prepared from mice lacking cdk5 there is elevated JNK3 activity in association with increased sensitivity to apoptosis. Moreover, the cdk5 inhibitor roscovitine can prevent the Akt kinase activating and neuronal survival promoting effects of neuregulins, a class of epidermal growth factor-like molecules (Li et al. 2003). Indeed, cdk5 directly phosphorylates the ErbB2 and ErbB3 neuregulin receptors. The proposed cdk5-involving mechanisms regulating neuronal death and survival are summarized in figure 2.

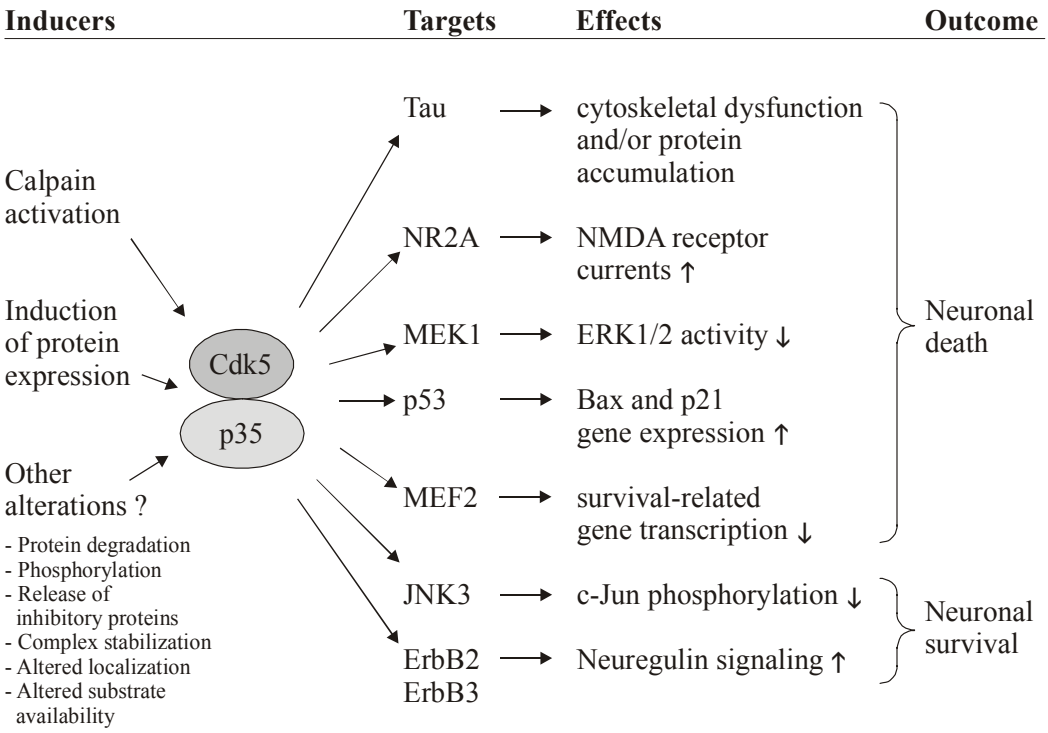

Figure 2. Summary of the proposed cdk5-involving mechanisms regulating neuronal death and survival. The figure shows the putative inducers of cdk5 alterations, immediate molecular targets of cdk5, the proposed downstream effects, and the final outcome.

3. AIMS OF THE STUDY

Previous studies have established the crucial role of cdk5 in the regulation of pivotal neurobiological events, and implicated deregulation of cdk5 in several neuropathological conditions. Detailed knowledge on the regulation of cdk5 will promote understanding of basic neurobiology, and strengthen the foundation of studies aiming at therapeutic interventions for neurological disorders. Neuronal loss is an important feature of several pathological conditions highlighting the need for research on the mechanisms of death of these largely irreplaceable cells. The aim of the present study was to investigate the regulation of cdk5, especially during the course of neuronal cell death, and also to evaluate the possible association between cdk5 and phosphorylation of the microtubule-associated protein tau.

The specific aims of the study were:

Study I: To explore how the expression of cdk5 and p35 proteins, as well as cdk5 activity and tau phosphorylation, are regulated during apoptotic neuronal death.

Study II: To investigate how calpain-mediated cleavage of p35 to p25 and phosphorylation of tau protein are affected during neuronal cell death induced by intracellular calcium concentration-elevating treatments.

Study III: To elucidate the contribution of different ionotropic glutamate receptors on the calpain-mediated cleavage of p35 in cultured neurons.

Study IV: To study how p35 phosphorylation can affect the proteolysis of the protein.

4. MATERIALS AND METHODS

4.1. Hippocampal neuronal cultures

Primary cultures of hippocampal neurons were isolated from 17-days-old Wistar rat embryos, and cultured essentially as previously described (Brewer et al. 1993). Pregnant rats were anesthetized with CO_2 and decapitated. The fetuses were removed and the hippocampi were dissected. The hippocampi were incubated for 10 min at +37 °C in phosphate-buffered saline (PBS) containing 10 mM glucose and 1 mg/ml bovine serum albumin (BSA), which had been supplemented with papain (0.5 mg/ml), DNase I (10 µg/ml), and $MgSO_4$ (2.4 mM). Thereafter the solution was replaced with PBS-glucose-BSA supplemented with DNase I (40 µg/ml) and $MgSO_4$, and then the hippocampi were triturated, and the suspension was allowed to settle for a few minutes. The supernatant was collected, and the trituration was repeated with a fresh solution. The supernatants were combined and centrifuged for 5 min at 150 g. The pellet was suspended in Neurobasal medium (Gibco) supplemented with 0.5 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5 % inactivated fetal bovine serum (FBS, HyClone). The cells were plated onto poly-D-lysine coated cell culture dishes at a density of 60000 cells/ cm^2, and maintained at +37 °C in a cell culture incubator in an atmosphere of 5 % CO_2. On the next day, the medium was replaced with fresh medium containing B27 supplement (Gibco) and lacking FBS. When the cells were planned to be cultured for 13 days before subjecting to experiments, half of the medium was replaced after 8 days of culture.

4.2. Cell culture models of neuronal death

Apoptosis was induced in hippocampal neurons by subjecting the cells to different apoptosis-inducing chemicals after 7 – 9 days in culture. The chemicals used included the protein phosphatase 1 and 2A inhibitor okadaic acid (10 nM, Calbiochem), the topoisomerase II inhibitor etoposide (5 µM, Calbiochem), the calcineurin inhibitor

cyclosporin A (10 μM, Calbiochem), and the lipid peroxidation product 4-hydroxynonenal (HNE, 20 μM, Calbiochem). The incubation times ranged from 6 to 24 hours.

Treatments of hippocampal neurons with glutamate agonists were performed after 13 days in culture, as in younger cultures the NMDA receptors are believed to be immature (Peterson et al. 1989, Cheng et al. 1999). In study III, also 6-day-old cultures were employed to evaluate the alterations in glutamate responses taking place during the maturation of neurons in culture. In study III, the glutamate agonists were added 15 min after the culture medium had been replaced, whereas in studies II and IV the agonists were added into the conditioned medium present on the culture dishes. The glutamate agonists used were glutamic acid (40 – 2000 μM, Sigma), NMDA (20 – 2000 μM, Sigma), kainic acid (5 – 1000 μM, Sigma), AMPA (100 – 1000 μM, Sigma), and 3,5-dihydroxyphenylglycine (DHPG, 100 – 1000 μM, Tocris). Exposure times were 30 min – 8 h in study II, 30 min in study III, and 60 min in study IV. The experiments with calcium ionophores were performed with hippocampal neurons cultured for 13 days, and the incubation times were between 40 and 45 minutes. The ionophores included ionomycin (1 – 16 μM, Calbiochem) and A23187 (1 – 20 μM, Calbiochem). The culture medium was supplemented with 2.5 mM $CaCl_2$ when the incubations with glutamate agonists or calcium ionophores were initiated, unless otherwise indicated.

In some experiments, the cultures were preincubated for 10 – 30 min with glutamate antagonists or different inhibitors. The glutamate antagonists used were the NMDA antagonist MK-801 (10 μM, Tocris), and the non-NMDA antagonists 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX, 100 μM, Sigma) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 100 μM, Sigma). The inhibitors employed were the calpain inhibitor MDL28170 (100 μM, Calbiochem), the L-type VSCC blocker nifedipine (200 μM, Sigma), the AMPA receptor desensitization inhibitor cyclothiazide (CTZ, 150 μM, Tocris), the kainate receptor desensitization inhibitor concanavalin A (Con A, 750 μg/ml, Sigma), the cdk5 inhibitor roscovitine (20 – 40 μM, Calbiochem), and the proteasome inhibitor lactacystin (5 μM, Calbiochem).

The key findings of the study were observed in at least two separate experiments. The reported protein and cell death analyzes were performed on the same dish of cells.

4.3. Preparation and manipulation of protein samples

4.3.1. Preparation of protein samples

The cell cultures were washed with PBS and scraped in protein lysis buffer containing 20 mM MOPS (3-[N-morpholino]propanesulfonic acid) (pH 7.2), 1 mM $MgCl_2$, 0.3 M NaCl, and 0.5 % Nonidet P-40, supplemented with Complete protease inhibitor cocktail (Roche). In studies III and IV, and in study II (when indicated) protein phosphatase inhibitors (50 mM NaF, 100 μM Na-orthovanadate, 5 mM Na-pyrophosphate, 5 mM EDTA) were added into the lysis buffer. The lysates were centrifuged at 13,000g for 20 minutes (+4 °C), and the supernatants were collected. The protein content of the samples was measured employing a DC Protein Assay kit (Bio Rad). When protein samples were isolated from rat brain tissue, the 1-day-old Wistar rat pups were sacrificed by decapitation, and the brain tissue (cerebral or cerebellar cortices) was homogenized in the protein lysis buffer, and processed as the cell culture samples described above.

4.3.2. Phosphatase treatment of protein samples

The protein samples were diluted 6 – 14 fold in the protein lysis buffer lacking protein phosphatase inhibitors. In study IV, 5 mM Na-orthovanadate was added into the diluted samples to prevent dephosphorylation of p35 occurring efficiently due to endogenous phosphatases. The samples were incubated for 1 h at 30 °C with 200-400 U of λ-protein phosphatase (λ-PPase, New England BioLabs) in 1× phosphatase buffer containing 50 mM Tris (pH 7.5), 0.1 mM EDTA, 5 mM dithiotreitol, and 0.01 % Brij 35, supplemented with 2 mM $MnCl_2$. A control reaction was carried out in the absence of λ-

PPase. The reaction was terminated by addition of electrophoresis sample buffer followed by heating of the samples for 5 min at 95 °C.

4.3.3. ATP treatment of protein samples

Incubation of protein samples with ATP was performed to study the phosphorylation of the p35 protein by endogenous kinases *in vitro*. Rat cortical homogenate was prepared in the protein lysis buffer in the absence of phosphatase inhibitors. The homogenate was incubated for 20 min – 3 h at 30 °C with 4 mM $MgCl_2$ in the presence or absence of 200 μM – 1 mM ATP. In some cases 50 mM NaF and 100 μM Na-orthovanadate were included into the incubation. The samples were analyzed by immunoblotting with an anti-p35 antibody, as will be described later.

4.3.4. Calpain treatment of brain homogenate

Rat cortical brain homogenate was prepared in the protein lysis buffer in the absence of phosphatase and protease inhibitors. The effect of protein phosphorylation on calpain-mediated p35 cleavage was studied by subjecting the brain homogenate first to an ATP treatment, followed by incubation for 20 min at 37 °C with 5 mM $CaCl_2$ in the presence or absence of purified calpain II (0.5 U, Calbiochem). The reaction was terminated by addition of electrophoresis sample buffer followed by heating of the samples for 5 min at 95 °C.

4.4. Analysis of cdk5, p35, and tau proteins by immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added onto equal amounts of protein samples (10-40 μg), and the samples were heated for 5 min at 95 °C. The proteins were resolved using 6 – 12 % sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), and blotted onto Hybond ECL nitrocellulose membranes (Amersham). Transfer efficiency and equal loading of the samples was

confirmed by staining of the blots with Ponceau S (Sigma). The membranes were blocked for 1 h in PBS containing 1 % BSA, 0.2 % non-fat dried milk, and 0.05 % Tween-20. Thereafter the blots were subjected for 1 h to primary antibodies diluted in the blocking buffer. Primary antibodies were used at the following dilutions: p35 (Santa Cruz, C-19), 1:500-1:1000; cdk5 (Santa Cruz, C-8), 1:1000; tau-5 (BioSource, against phosphorylation-independent epitope of tau), 1:12000; AT8 (Innogenetics, epitope around phosphorylated residue 202 of human tau), 1:2000-1:3000; AT180 (Innogenetics, tau phosphorylated at residue 231), 1:2000; AT270 (Innogenetics, tau phosphorylated at residue 181), 1:12000; pT212 (BioSource, tau phosphorylated at residue 212), 1:8000; pS404 (BioSource, tau phosphorylated at residue 404), 1:8000. The membranes were washed three times for 5 min in PBS containing 0.05 % Tween-20, and thereafter incubated for 1 h with peroxidase-conjugated anti-rabbit (Amersham, 1:2000-1:4000), or anti-mouse (Amersham, 1:2000-1:4000) antibodies diluted in the blocking buffer. The membranes were washed as above, and the bound antibodies were visualized by enhanced chemiluminescence reaction (Pierce) and film detection (Hyperfilm ECL, Amersham).

4.5. Cdk5 activity assay

Cdk5 kinase activity was measured essentially as previously described (Saito et al. 1998). Fifty µg of protein in 100 µl of protein lysis buffer was incubated with 3 µl of anti-cdk5 antibody (Santa Cruz, C-8) for 4 h at +4°C with constant mixing. Twenty µl of protein A-Sepharose suspension (Amersham) was added, and the incubation was continued overnight. The Sepharose was collected by centrifugation (30 sec, 13000 g), and washed twice with washing buffer containing 10 mM MOPS (pH 7.2), 1 mM $MgCl_2$, 1 mM EGTA, 5 mM EDTA, 50 mM NaF, 100 M Na-orthovanadate, and 5 mM Na-pyrophosphate, and once with assay buffer containing 10 mM MOPS (pH 7.2), and 1 mM $MgCl_2$. A kinase reaction was carried out by adding 50 µl of assay buffer with 0.1 mM ATP, 0.4 mg/ml histone H1, and 0.1 mCi/ml [γ-32P]ATP onto the Sepharose, and incubating the samples for 30 min at 30 °C. The reaction was terminated by addition of electrophoresis sample buffer followed by heating of the samples for 5 minutes at 95 °C. The proteins were separated employing a 12 % SDS-PAGE gel, and

the gel was dried. Radioactivity on the gel was detected by a Storm PhosphorImager (Molecular Dynamics), and the pixel volumes of the radioactive histone H1 bands were quantified with ImageQuaNT software (Molecular Dynamics).

4.6. Characterization of cell death

4.6.1. Analysis of cell morphology

The cellular morphology was evaluated to obtain an overall impression of the extent of neuronal damage. Photographs were taken with a camera attached to an inverted microscope immediately prior to collection of protein samples. In study II the morphological changes associated with neuronal damage were graded as described in figure 3.

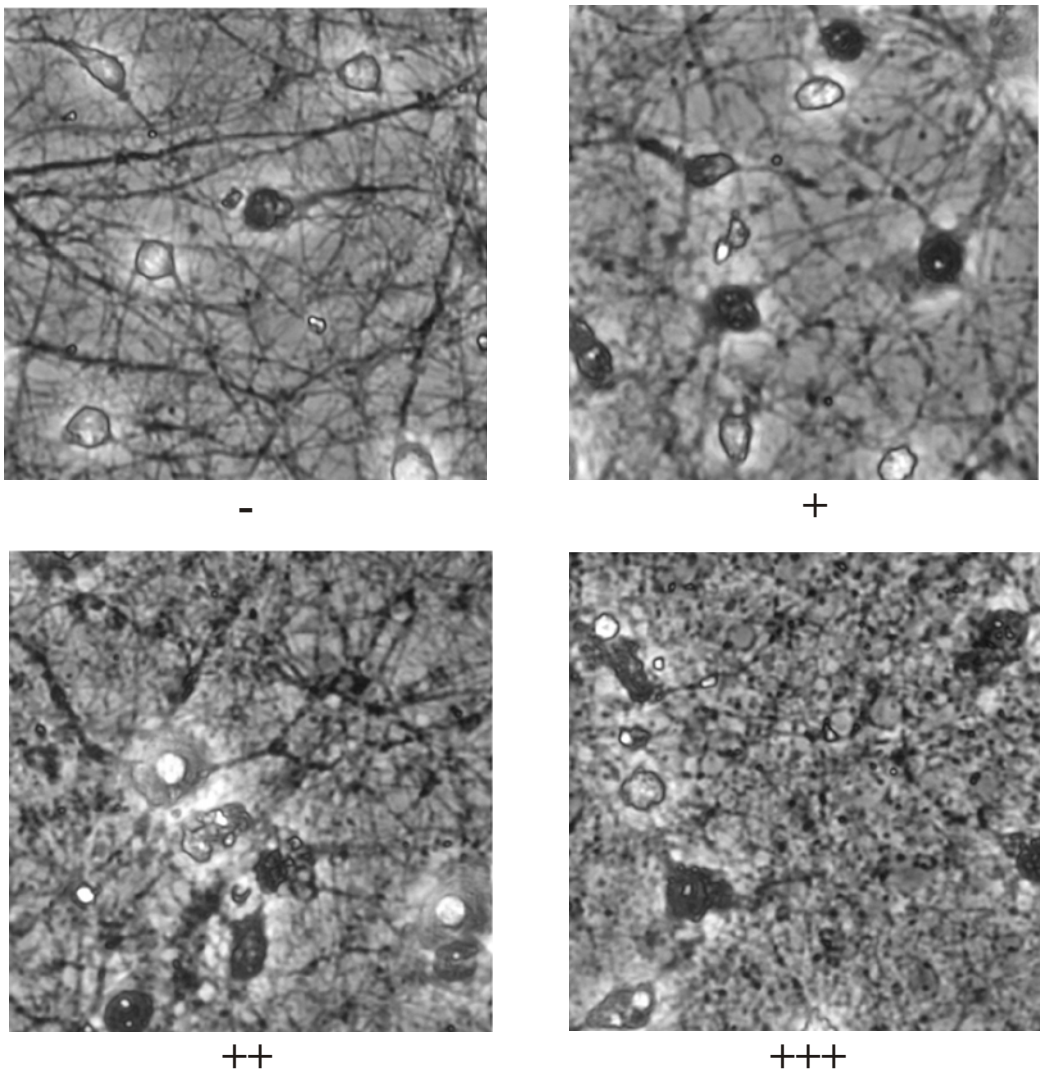

Figure 3. Grading of neuronal damage by morphological analysis. The morphology of neuritic network was graded to be either unchanged ("-" = no changes whatsoever detectable when compared to an untreated control culture), or to have minor changes ("+" = local swellings of neurites clearly detectable), moderate damage ("++" = the neurites clearly fragmented), or extensive damage ("+++" = even the thickest processes fragmented).

4.6.2. Assessment of LDH release

A sample of the cell culture medium was collected to analyze the release of lactate dehydrogenase (LDH) to the culture medium upon cell lysis. The medium sample was clarified by centrifugation (13000 g, 30 sec), and LDH activity was analyzed with the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) according to the

manufacturer's instructions. The percentage of cell death was calculated by designating LDH release in an untreated control culture as zero percent cell death, and LDH release in a detergent lysed culture as 100 percent cell death.

4.6.3. Measurement of cellular ATP levels

The neuronal cultures were washed with PBS, and the cells were lysed in Somatic Cell ATP Releasing Reagent (Sigma). The ATP levels in the samples were measured using an enzymatic luciferase-based Bioluminescent Somatic Cell Assay kit (Sigma) according to the manufacturer's instructions.

4.6.4. Caspase-3 activity assay

Caspase-3 activity was examined by measuring the amount of a fluorescent product released from a synthetic substrate of caspase-3, essentially as suggested by the manufacturer of the substrate. In brief, 255 µl of a solution containing 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) (pH 7.5), 10 % glycerol, 2 mM dithiothreitol, and 20 µM Ac-DEVD-AMC (Pharmingen) was added onto 25 µg of a protein sample (in 25 µl volume) in the wells of a black 96-well microtiter plate. The plate was mixed, and after 60 min in +37 °C, the fluorescence was measured with a Victor 1420 Multilabel Counter (Wallac) using 355 nm excitation and 460 nm emission wavelengths.

4.6.5. Analysis of αII-spectrin breakdown products

Analysis of the breakdown products of αII-spectrin was used to detect calpain-protease activity. αII-Spectrin was analyzed from cell lysates by immunoblotting as described above. The αII-spectrin primary antibody (Santa Cruz, C-20) was diluted 1:100, and the peroxidase-conjugated anti-goat antibody (Boehringer-Mannheim) was diluted 1:2000. Calpain cleaves αII-spectrin from two different sites leading to C-terminal fragments

with molecular weights of 145 kDa and 150 kDa. It should be noted that also caspase-3 can generate a cleavage product with a molecular weight of 150 kDa (Wang 2000).

5. RESULTS

5.1. Cdk5 and p35 proteins as well as cdk5 activity and tau phosphorylation are downregulated during neuronal apoptosis (I)

Apoptotic cell death was induced in rat hippocampal neuronal cultures by subjecting the cells to different apoptosis-inducing compounds. Neuronal damage was evaluated microscopically, and activation of the apoptotic cascade was assessed by measuring the activity of caspase-3, a central effector caspase. Activity of calpain, a protease activated in various necrotic and apoptotic conditions, was detected by examining the level of calpain-generated cleavage products of endogenous αII-spectrin.

Okadaic acid induced morphologically visible neuritic damage after 6 hours of treatment, whereas the first signs of damaged neuritic network were seen after 12 h in neurons treated with etoposide, cyclosporin A, and HNE. All the compounds induced caspase-3 activation, ranging from 6 to 20-fold greater activity when compared to an untreated control culture (Fig. 1, study I). The level of the 145 kDa calpain-type cleavage product of αII-spectrin was low and only detected in cells subjected to cyclosporin A or okadaic acid treatment.

The level of p35 protein was reduced with okadaic acid and HNE after 6 h, and with etoposide and cyclosporin A after 12 h of treatment (Fig. 2, study I). The p25 levels were also reduced early during the course of apoptosis. However, the levels of cdk5 protein remained relatively constant until becoming reduced at 24 h. HNE provided an exception by inducing an early decrease in the cdk5 level. Cdk5 activity was generally reduced during the course of cell death. However, the correlation of cdk5 activity with p35, p25, and cdk5 protein levels was not obvious in all conditions. We also monitored the phosphorylation of tau protein, a substrate of cdk5, during the course of cell death. Immunoblotting with the AT8 antibody was used as the phosphorylation-dependent epitope of this antibody has been suggested to be especially relevant with respect to cdk5-mediated phosphorylation (Patrick et al. 1999, Hashiguchi et al. 2002, Hamdane et

al. 2003). Tau phosphorylation was generally slightly reduced during the early phase of neuronal death (6 – 12 h), but later phosphorylation was partially restored. Okadaic acid induced a substantial increase in tau phosphorylation, probably because tau is a substrate of the protein phosphatases inhibited by okadaic acid. An additional tau band (47 kDa) was detected in okadaic acid and etoposide treated cells. When a longer exposure time of the blot was used, the additional band was observed also in cultures subjected to cyclosporin A or HNE (data not shown). The appearance of this band peaked at the time of caspase-3 activation. Indeed, the 47 kDa tau isoform may represent a caspase cleavage product, as tau is known to be a substrate for caspases (Canu et al. 1998, Ko et al. 2000, Krishnamurthy et al. 2000).

5.2. Calpain-mediated cleavage of p35 to p25 during neuronal cell death is not associated with tau hyperphosphorylation (II)

Rat hippocampal neuronal cultures were subjected to toxic treatments known to increase the concentration of intracellular calcium. The relationship between the calpain-mediated cleavage of p35, cdk5 activity, and tau phosphorylation was studied during the course of cell death. Glutamate induced a prominent cleavage of p35 to p25 already after 30 min of treatment, whereas the protein level of cdk5 remained relatively constant for 4 h (Fig. 1A, study II). Cdk5 activity was elevated in association with the increased p25 levels after a short (30 min – 2 h) glutamate exposure, whereas no increase in cdk5 activity was detectable after longer incubations (4 – 8 h). As activation of cdk5 has been suggested to induce hyperphosphorylation of tau, we also evaluated the relationship between cdk5 activity and tau phosphorylation in glutamate treated cells. Immunoblotting with a phosphorylation-independent tau antibody (Tau-5) revealed a transient appearance of two fast-migrating tau bands. These bands likely represent dephosphorylated forms of tau, as similar isoforms were generated when a protein sample from cultured neurons was exposed to λ-protein phosphatase (Fig. 1B, study II). In agreement with these findings, tau phosphorylation was found to be reduced to a varying extent in glutamate treated neurons with five different phosphorylation-dependent tau antibodies recognizing epitopes phosphorylatable by cdk5 (Fig. 1A, study II). The calpain inhibitor MDL28170 prevented the glutamate-induced cleavage of p35

to p25, as well as the cleavage of αII-spectrin, another calpain substrate (Fig. 1C, study II). However tau phosphorylation remained unaffected suggesting that calpain-mediated processes, such as cleavage of p35 to p25, do not contribute to tau phosphorylation in these conditions. In general, the first evidence for altered p25 production, cdk5 activity, or tau phosphorylation was observed before significant alterations in neurite morphology or loss of membrane integrity (LDH release) in glutamate treated cultures (Fig. 1A, study II). However, cellular ATP levels displayed a 20 % and 45 % decrease in cultures incubated for one hour with 40 μM and 200 μM glutamate, respectively, indicating an early disturbance of the energy metabolism.

NMDA, an agonist of the NMDA class of glutamate receptors, also resulted in rapid (30 min) cleavage of p35 to p25, and decreased tau phosphorylation (Fig. 2, study II). These changes also appeared well before any significant changes in neuritic morphology or LDH release were apparent (Fig. 2, study II), whereas the amounts of cellular ATP were decreased by 19 % and 25 % with 80 and 400 μM NMDA, respectively, when compared to an untreated culture after 1 h of incubation.

Cleavage of p35 to p25 was also observed in cultured neurons treated with the calcium ionophores ionomycin or A23187 (Fig. 3A and 3C, study II). Decreased tau phosphorylation was also detected in these conditions (Fig. 3A and 3D, study II). Again, in the cultures treated with the calcium ionophores, the cellular ATP levels were decreased (Fig. 3B and 3E, study II). In contrast to glutamate and NMDA treatments, the calcium ionophores affected the p25 level and tau phosphorylation only under conditions where morphological evidence of cellular damage was apparent (Fig. 3A and 3D, study II).

5.3. Both NMDA and non-NMDA receptors are able to mediate glutamate-induced cleavage of p35 to p25 in cultured neurons (III)

The role of different calcium-permeable ionotropic glutamate receptors in the induction of calpain-mediated p35 cleavage was elucidated during the early phase of glutamate excitotoxicity in cultured rat hippocampal neurons. A 30-minute treatment of the

cultures with glutamate and NMDA, as well as with the non-NMDA receptor agonists kainic acid and AMPA, induced prominent cleavage of p35 to p25 (Fig. 1A, study III). The term “non-NMDA receptors” is commonly used collectively to refer to the kainate and AMPA receptors, as the receptors have significant structural similarity and overlapping pharmacological properties. The cleavage of p35 was prevented when the cultures were subjected to the glutamate agonists in the presence of the cell-impermeable calcium chelator ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (Fig. 2A, study III). This suggests that the p35 cleavage-inducing calcium mainly originates from the extracellular pool, which is further supported by the increased p35 cleavage in the presence of elevated extracellular calcium level. Moreover, DHPG, an agonist of the metabotropic group I glutamate receptors, did not promote p35 cleavage (Fig. 1B, study III), although these receptors have been linked to calcium release from intracellular sources (Sugiyama et al. 1987).

Neuronal responses to glutamate are known to undergo significant changes during maturation of neurons in culture (Cheng et al. 1999). Although glutamate, NMDA, and kainic acid were all able to induce p35 cleavage to p25 in the 13-day-old hippocampal neuronal cultures used above, in the more immature 6-day-old cultures, the effect of glutamate was barely detectable, and NMDA had no effect (Fig. 1C, study III). Nevertheless, kainic acid caused cleavage of p35 also in these immature cultures.

When studying the contribution of different classes of glutamate receptors on the p35 cleavage induced by glutamate, it was observed that MK-801, an antagonist of the NMDA receptor, prevented the p35 cleavage to p25 in glutamate-treated neurons (Fig. 2B, study III). However, the non-NMDA antagonists NBQX and CNQX did not block the glutamate-induced p35 cleavage. This suggests that the NMDA receptors are the predominant mediator of glutamate-induced p35 cleavage under normal culture conditions. As expected, the effect of NMDA was blocked by MK-801, whereas the action of kainic acid was prevented completely by NBQX and partially by CNQX. Also nifedipine, a blocker of the L-type voltage-sensitive calcium channels, partially prevented the effect of all of the glutamate agonists studied.

Inhibitors of AMPA and kainate receptor desensitization are the most selective pharmacological tools available to make a distinction between these non-NMDA receptors. When the NMDA receptors in the neuronal cultures were blocked by MK-801, glutamate alone was inefficient, but with CTZ, a selective inhibitor of AMPA receptor desensitization, glutamate induced p35 cleavage to p25 (Fig. 3A, study III). This suggests that desensitization of AMPA receptors may prevent glutamate from inducing p35 cleavage via AMPA receptor activation in these cultures. Concanavalin A (Con A), an inhibitor of kainate receptor desensitization, had a barely detectable effect. When a low concentration of the non-NMDA receptor agonist kainic acid was applied together with CTZ or Con A, both desensitization inhibitors promoted p35 cleavage (Fig. 3B, study III). This implies that the effect of kainic acid on p35 cleavage may be mediated through both AMPA and kainate receptors. Also in the absence of agonists, CTZ was able to induce p35 cleavage (Fig. 3A and 3B, study III), possibly indicating AMPA receptor activation by glutamate constantly released from the cultured neurons. Accordingly, previous studies have shown that endogenous glutamate is able to induce non-NMDA receptor activity, a property which is strongly enhanced by CTZ (Vignes 2001).

5.4. Phosphorylation of p35 affects its proteasomal degradation and calpain-mediated cleavage to p25 (IV)

Protein samples prepared in the absence of phosphatase inhibitors from newborn rat cortical or cerebellar tissue, or from cultures of rat hippocampal neurons, contained p35 protein migrating as a single band in immunoblot (Fig. 1A, study IV). However, when protein samples were extracted in the presence of phosphatase inhibitors, p35 displayed a mobility shift on SDS-PAGE, leading to a doublet (or triplet) band in the immunoblot. This doublet band was converted to a single band when cortical brain lysate was incubated with λ-protein phosphatase (Fig. 1B, study IV). Also, incubation of brain lysate with ATP resulted in slower electrophoretic migration of p35 (Fig. 1C, study IV). These results indicate that the upper band of the p35 doublet represents p35 in a phosphorylated form. Treatment of rat hippocampal neuronal cultures with roscovitine, an inhibitor of cdk5, led to loss of the phosphorylated upper p35 band in the

immunoblot (Fig. 2A, study IV). Roscovitine also inhibited the phosphorylation of p35 in rat cortical brain homogenate treated with ATP (Fig. 2B, study IV). Collectively the above data suggest that p35 is phosphorylated in rat brain tissue to a significant extent, and that the main p35-phosphorylating kinase is cdk5.

Previous studies have suggested that phosphorylation of p35 affects its degradation by the proteasome in cultured cells (Patrick et al. 1998, Saito et al. 1998). Indeed, the proteasome inhibitor, lactacystin, prevented the increased degradation of p35 in neuronal cultures treated with the protein phosphatase inhibitor okadaic acid (Fig. 4, study IV), consistent with phosphorylation dependent degradation of p35 by the proteasome. To study further the p35 phosphorylation events occurring in the presence of okadaic acid, brain homogenate from rat cortex was treated with okadaic acid, in combination with ATP for 30 min or 3 h. In brain homogenate, ATP alone induced a mobility shift in p35 (Fig. 3A, study IV). Addition of okadaic acid together with ATP resulted in a p35 band that migrated slower than the band generated by ATP alone. This band likely represents p35 phosphorylated at an additional site. On the top of this main band there were multiple less intense bands separated from each other by a distance corresponding to the phosphorylation induced shift. These multiple bands probably represent p35 phosphorylated at a varying number of additional sites. Roscovitine, an inhibitor of cdk5, prevented the formation of the multiple p35 bands, as well as changing the mobility of the main phosphorylated p35 band in brain homogenate treated with ATP and okadaic acid. However, roscovitine did not inhibit all phosphorylations of p35. The data suggest that in brain homogenate, ATP and okadaic acid induce p35 phosphorylation by cdk5, and by a yet unidentified other kinase(s). The decreased intensity of the p35 band in these experiments was not due to proteolytic degradation of p35, as a phosphatase treatment of brain homogenate after a reaction with ATP and okadaic acid restored the intensity of the p35 band (Fig. 3C, study IV).

Calpain is another p35-cleaving protease, and it is responsible for converting p35 to a p25 fragment. We investigated whether the phosphorylation of p35 could modulate also this cleavage. In brain homogenates, p35 phosphorylation was first induced with ATP and okadaic acid, and thereafter the homogenates were treated with calcium to activate

endogenous calpain. Addition of calcium to brain homogenate induced the formation of p25, which was augmented when purified calpain II was included (Fig. 5B, study IV). Extensive phosphorylation of p35 as a result of ATP and okadaic acid treatment led to a diminished amount of p25 production. Inhibition of cdk5-dependent phosphorylation by roscovitine partially restored the intensity of p25, suggesting that cdk5-mediated phosphorylation could be involved in the phosphorylation-dependent reduction of p35 cleavage. However, in glutamate treated rat hippocampal neuronal cultures, roscovitine did not alter the amount of p25 produced (Fig. 5A, study IV).

6. DISCUSSION

6.1. Regulation of cdk5 during neuronal cell death

Cdk5 has been suggested to regulate several different molecular and cellular factors involved in neuronal cell death. Events occurring during the course of neuronal death could lead to alterations in cdk5, which might amplify the process. In this study, we evaluated the changes in the regulation of cdk5 in primary rat hippocampal neuronal cultures subjected to various toxic conditions. Although the objective was to mimic the processes of neuronal death occurring in different human neuropathological conditions, it should be noted, that the cell culture conditions employed, as well as the resulting cellular responses, are likely to differ from the corresponding *in vivo* events. It is obvious that the results obtained with neuronal cell cultures need to be supplemented by evidence from other experimental model systems or human brain material, whenever possible. The analytical methods used in this study mainly involved different types of protein analyses from lysates of cultured neurons. Therefore, the results reflect the overall cellular responses during neuronal death, whereas the possible events taking place only in a minority of cells, or only in specific subcellular locations may not be detected.

Induction of cell death in neuronal cultures. Neuronal death by apoptosis has been implicated in several neuropathological conditions (Friedlander 2003). In this study, apoptotic cell death was induced in neuronal cultures by subjecting the cells to different types of commonly used pro-apoptotic compounds. Etoposide (an inhibitor of topoisomerase II), okadaic acid (an inhibitor of protein phosphatases 1 and 2A), cyclosporin A (an inhibitor of calcineurin), and HNE (a product of lipid peroxidation) have been observed to induce neuronal cell death showing apoptotic features, such as apoptotic nuclear morphology and DNA degradation (Nakajima et al. 1994, Cagnoli et al. 1996, McDonald et al. 1996, Kruman et al. 1997, Bezvenyuk et al. 2000, Kaminska et al. 2001, Kim et al. 2001, Rabacchi et al. 2004). Induction of caspase-3 activity confirms that these compounds did activate the apoptotic cascade in our study. Calpain,

a calcium-activated protease more closely associated with necrotic cell death, did not appear to be significantly activated in these conditions, as the level of calpain-type cleavage products of αII-spectrin remained low. Cell death was also induced by treating cultured neurons with compounds known to evoke prominent calcium influx, i.e. calcium ionophores and glutamate agonists. Glutamate excitotoxicity is believed to be involved in neuronal loss in both acute and chronic neuropathological conditions (Lipton and Rosenberg 1994). Glutamate treatment of neuronal cultures provides a good tool to study excitotoxicity, however the treatment may more closely model acute excitotoxicity than chronic conditions. Although we studied the effects of glutamate agonists under excitotoxic conditions, the results can be cautiously considered when evaluating the post-synaptic effects of glutamate under physiological conditions. In neuronal cultures, glutamate excitotoxicity can be alleviated by inhibiting either calcium influx or calpain activation (Choi 1987, Brorson et al. 1995a). We detected calpain-type cleavage products of αII-spectrin in glutamate-treated neurons, and their appearance could be prevented by a calpain inhibitor, showing activation of calpain under these conditions.

Cdk5 and p35 protein levels during neuronal death. During the course of apoptotic cell death, the protein levels of p35, p25, and cdk5 were decreased. A similar tendency was observed with several different pro-apoptotic treatments, which suggests that the results may reflect a general response to this type of condition. The first signs of decreased p35 and p25 levels generally appeared relatively early during the course of apoptotic cell death i.e. in cells showing no or only minor signs for neuritic damage as characterized by morphological evaluation. Okadaic acid provoked a particularly early and prominent downregulation of p35 and p25 proteins. However, this likely reflects the effect that p35 phosphorylation has on the proteasomal degradation of this protein (Saito et al. 1998) rather than being a consequence of cell death-associated phenomena. The reduction of cdk5 protein expression during apoptosis was mainly restricted to severely damaged cells. Accordingly, the cdk5 level was decreased also in neurons profoundly affected by glutamate treatment. The mechanisms responsible for the reductions in p35, p25, and cdk5 protein levels in these conditions remain unknown.

Studies regarding the levels of cdk5 and p35 proteins during experimentally induced neuronal death have led to somewhat inconsistent conclusions. The variability of changes in these protein levels during neuronal death may be explained for instance by dissimilar responses to different types of cell death-inducing stimulus. In cultured neurons, the protein levels of cdk5 or p35/p25 have been reported to be decreased due to oxidative damage (Shirvan et al. 1998, Egana et al. 2003) or hypoxia/reoxygenation (Vartiainen et al. 2002), but the levels are increased following exposure to colchicine (Canudas et al. 2004) or staurosporine (Zhang et al. 2004a). In experimental animals, the immunoreactivities for cdk5 and p35 are frequently induced in cell bodies and nuclei during neuronal death characterized by activation of apoptotic mechanisms (Henchcliffe and Burke 1997, Zhang et al. 1997, Neystat et al. 2001). The upregulation of cdk5 and p35 is especially detected in cells containing fragmented DNA, or apoptotic nuclear changes (Henchcliffe and Burke 1997, Zhang et al. 1997, Neystat et al. 2001) suggesting that induction of these proteins may be a late event of apoptosis. Activation of gene expression would be a straightforward explanation for the upregulation of the proteins in these conditions. The cdk5 and p35 mRNA levels have been determined during apoptotic neuronal death in the substantia nigra following a striatal lesion, however no alterations were detected (Neystat et al. 2001). Also, following ischemia the immunostaining for cdk5 and p35 is increased within 1 h in the neuronal cell bodies and nuclei in the affected area (Hayashi et al. 1999) revealing that these kinds of changes can occur very rapidly. Since cdk5 protein is normally predominantly expressed in axons (Tsai et al. 1993, Ino and Chiba 1996, Matsushita et al. 1996, Neystat et al. 2001), the increased levels of cdk5 in neuronal cell bodies could alternatively indicate altered intracellular localization of the protein in stressful conditions. This kind of phenomenon has been observed to take place during kindling progression (Tomizawa et al. 2000, Cai et al. 2001).

Calpain-mediated cleavage of p35 during neuronal death. Increased cleavage of p35 to p25 by the calcium-activated calpain protease has been frequently observed in cultured neurons subjected to toxic conditions (Kusakawa et al. 2000, Lee et al. 2000, Nath et al. 2000, Canudas et al. 2004), and in experimental animal models of neurological disorders (Lee et al. 2000, Nath et al. 2000, Nguyen et al. 2001, Bu et al. 2002, Smith et

al. 2003). Calpain-mediated p35 cleavage has been suggested to deregulate cdk5, which may promote neuronal death (Patrick et al. 1999, Cruz et al. 2003, Hamdane et al. 2003). However, we detected no signs of p35 cleavage to p25 during apoptotic neuronal death, which suggests that p35 cleavage is not an universal event in neuronal apoptosis, at least in the early phase in the models studied. The lack of p35 cleavage induction was in good agreement with the virtual absence of calpain-type cleavage of αII-spectrin. It is possible that calpain-induced deregulation of cdk5 may be restricted to conditions where calcium influx is particularly intense. One good candidate as an inducer of p35 cleavage in both physiological and pathological conditions is the major excitatory neurotransmitter glutamate. We observed that glutamate treatment of neuronal cultures led to rapid calpain-mediated cleavage of p35 to p25, which preceded the appearance of signs for cellular damage, i.e. morphological alterations or leakage of the intracellular enzyme LDH. Therefore the calpain-induced changes in the function of cdk5 may be able to modulate even the relatively early events of glutamate excitotoxicity. The effect of NMDA was similar to that of glutamate, whereas calcium ionophores produced p35 cleavage only in association with severe neuritic damage.

Pharmacological agents specifically modulating different glutamate receptors constitute a promising, although challenging, approach to dampen neuropathological processes. This approach requires a detailed knowledge on the role of these receptors in activating various downstream signaling pathways. We studied the role of different ionotropic glutamate receptors in the induction of calpain-mediated p35 cleavage in more detail during the very early phase of glutamate excitotoxicity in neuronal cultures. The experiments with different glutamate agonists suggest that both NMDA and non-NMDA classes of glutamate receptors possess the capability to activate p35 cleavage, in a manner dependent on extracellular calcium. In mature neuronal cultures, the glutamate-induced p35 cleavage was predominantly mediated via the NMDA receptors, although also the L-type VSCCs had a significant role. However, in more immature developing cultures, the non-NMDA receptors appeared to have more potential to activate p35 cleavage than the NMDA receptors. The changes in the capability of NMDA to induce p35 cleavage may be a consequence, for instance, from the changes

occurring in NMDA receptor subunit expression during maturation of neurons in culture (Cheng et al. 1999).

Both NMDA and non-NMDA receptors are able to mediate glutamate excitotoxicity as well as calpain activation. The ionotropic glutamate receptor class preferentially producing excitotoxicity may vary e.g. depending on the neuronal type (Brorson et al. 1995a). The calcium-permeable NMDA receptors have been traditionally considered of special importance with respect to excitotoxicity. However, the AMPA and kainate receptors can also be calcium-permeable, when a specific subunit of the receptor is missing, or present in a rare form produced from unedited mRNA (Dingledine et al. 1999, Tanaka et al. 2000, Arundine and Tymianski 2003). Additionally, activation of all of the ionotropic glutamate receptor classes evoke membrane depolarization, which contributes to the secondary opening of the VSCCs. As some signaling molecules are physically co-localized with specific glutamate receptors, the actual route of calcium influx may be more important than the extent of calcium influx in determining which intracellular excitotoxic pathways are activated (Sattler et al. 1998, Arundine and Tymianski 2003).

Ischemic damage in experimental animals represents a relevant *in vivo* model of neuronal death, having a significant component of excitotoxicity. In ischemia, antagonists of both NMDA and non-NMDA receptors reduce neuronal loss in the hippocampus, when administered before or shortly after the insult (Simon et al. 1984, Buchan et al. 1991). Recently, the calpain-mediated cleavage of p35 following ischemia was found to depend on activation of the non-NMDA receptors (Wang et al. 2003). The results of the study are compatible with a sequence of events, where activation of the non-NMDA receptors leads to cleavage of p35 to p25, and subsequent cdk5-dependent phosphorylation of the NR2A subunit of the NMDA receptor. This enhances the flow of ions through the NMDA receptors, and thereby promotes cell death of hippocampal CA1 neurons. Downregulation of the calcium permeability-preventing GluR2 subunit of the AMPA receptors takes place during ischemia, and this has been suggested to promote non-NMDA dependent neuronal death (Pellegrini-Giampietro et al. 1992). Interestingly, virus-mediated overexpression of GluR2 reduces neuronal death and p35

cleavage during ischemia (Liu et al. 2004). Our results provide further evidence that activation of the non-NMDA receptors contributes to p35 cleavage. The data with nifedipine suggest that also the L-type VSCCs may be involved in the non-NMDA receptor induced p35 cleavage.

Desensitization of the non-NMDA receptors is another feature possibly affecting the potential of these receptors to mediate glutamate excitotoxicity (Frandsen and Schousboe 2003). For instance, in cerebellar neuronal cultures, the selective vulnerability of Purkinje cells to excitotoxicity has been proposed to arise from the desensitization properties of the AMPA receptors expressed in these neurons (Brorson et al. 1995b). Our experiments with desensitization inhibitors of non-NMDA receptors revealed that the non-NMDA receptor-activated p35 cleavage was mediated via the AMPA receptors in the hippocampal neuronal cultures. The results also imply that the desensitization properties of AMPA receptors could significantly modulate the role of these receptors in the activation of p35 cleavage.

Cdk5 activity during neuronal death. The cdk5 activity was generally reduced during neuronal apoptosis thus paralleling the decreased amounts of cdk5, p35, and p25 proteins. However, the protein levels appeared to predict the activity of cdk5 only partially, possibly due to the involvement of other factors in the regulation of the kinase activity. These other factors could involve changes in cdk5 phosphorylation, or altered cdk5-p35 complex stability, as previously reported in neurons subjected to stressful conditions (Alvarez et al. 2001, Zambrano et al. 2004). In glutamate-treated neuronal cultures, the calpain-mediated cleavage of p35 was associated with increased cdk5 activity. This is consistent with the increased ability of p25 to activate cdk5 when compared to p35 (Amin et al. 2002). Nevertheless, following longer incubation periods with glutamate (4 – 6 h), the alterations in the cdk5 activity were not closely related to the protein levels of p35, p25, and cdk5.

Increased activity of cdk5, or the qualitative changes in the function of cdk5 induced by calpain-mediated p35 cleavage, have been suggested to promote the process of neuronal cell death. Our data imply that glutamate could be a potent activator of cdk5-involving

mechanisms of neuronal death. However, it is difficult to evaluate the significance of decreased cdk5 activity detected during the process of neuronal apoptosis. It can be hypothesized that the decline in the activity of cdk5 during neuronal apoptosis could reduce the ability of cdk5 to support critical neuronal functions.

6.2. Regulation of p35 proteolysis by phosphorylation

Previous studies suggest that p35 is a phosphoprotein (Lew et al. 1994, Tsai et al. 1994). In contrast to phosphorylation of cdk5, phosphorylation of p35 does not seem to influence its ability to activate cdk5 (Saito et al. 2003). Phosphorylation of cyclins often regulates the degradation of these proteins (Obaya and Sedivy 2002). Accordingly, p35 phosphorylation could affect the function of cdk5 by modulating the proteolysis of p35.

We analyzed p35 phosphorylation by monitoring the phosphorylation-induced retardation in the electrophoretic mobility of the protein by immunoblotting. A significant proportion of p35 was detected in a phosphorylated state in the cerebral cortex and cerebellum of newborn rats as well as in hippocampal neuronal cultures. The phosphorylation of p35 in neuronal cultures and in ATP-treated brain homogenates was sensitive to roscovitine, which is a selective inhibitor of cdk5, cdc2, and cdk2 (Meijer et al. 1997). As the expression of the cell cycle-associated cyclin-dependent kinases cdc2 and cdk2 become downregulated during neuronal differentiation (Hayes et al. 1991, Freeman et al. 1994, Shirvan et al. 1998, Courtney and Coffey 1999), the roscovitine-sensitive kinase responsible for p35 phosphorylation in these conditions is most likely to be cdk5. This is also supported by the observations showing phosphorylation of p35 *in vitro* by purified cdk5 (Lew et al. 1994, Tsai et al. 1994, Saito et al. 2003).

Previously p35 has been found to be ubiquitinated, and degraded in a manner dependent on phosphorylation of p35, and on the proteasome (Patrick et al. 1998). In cultured neurons the protein phosphatase inhibitor, okadaic acid, induces degradation of p35 (Saito et al. 1998). This can be prevented by a proteasome inhibitor consistent with phosphorylation-dependent degradation of p35 by the proteasome. Our data with hippocampal neuronal cultures treated with okadaic acid in the presence of lactacystin

confirm this finding. The cdk5-mediated phosphorylation of p35 has been suggested to promote proteasomal degradation of p35 (Patrick et al. 1998). However, the finding that a significant proportion of p35 exists in a cdk5-phosphorylated state in cultured neurons, may indicate that phosphorylation of p35 by cdk5 alone is not a sufficient signal for proteasomal degradation of p35. With respect to the p35 degradation-promoting effect of okadaic acid, this may be caused by additional phosphorylation of p35 by another kinase. In ATP-treated brain homogenate, okadaic acid induced phosphorylation of p35 at two sites, whereas ATP alone caused p35 phosphorylation at one site only. One of these two sites is likely phosphorylated by cdk5, whereas the other site appears to be phosphorylated by an unknown roscovitine-insensitive kinase. Phosphorylation of p35 by these kinases could provide a means to regulate p35 proteolysis, and thereby the function of cdk5, in physiological situations. Aberrations in the ubiquitin-proteasome system have been implicated in neurodegenerative disorders, such as PD and AD (Ciechanover and Brundin 2003). Currently there are no reports indicating that the proteasomal dysfunction in these disorders would be associated with a general accumulation of p35. Nevertheless, the proteasomal alterations might specifically delay the degradation of the phosphorylated p35 species, thereby affecting the possible regulation of cdk5 involving p35 phosphorylation.

We also studied the effect of p35 phosphorylation on the calpain-dependent cleavage of p35 to p25. In brain homogenate, phosphorylation of p35 induced by pre-incubation with ATP and okadaic acid reduced the amount of calcium-activated calpain-dependent cleavage of p35. The cdk5 inhibitor roscovitine partially prevented the effect of the phosphorylation reaction on the calpain-mediated p35 cleavage. However, roscovitine did not affect the calpain-mediated cleavage of p35 induced by glutamate in neuronal cultures. Based on these results, phosphorylation of p35 can reduce its calpain-mediated cleavage, whereas the role of cdk5-dependent p35 phosphorylation in this process remains elusive. The study by Saito et al. (2003) confirms that phosphorylation of p35 indeed reduces the calpain-mediated cleavage of p35 *in vitro*, and provides additional evidence for a role for cdk5-induced p35 phosphorylation in this process. Their data further suggest that phosphorylation and proteolysis of p35 is developmentally regulated. p35 in fetal rat brains was highly phosphorylated and susceptible to

proteasomal degradation, whereas in adult brains p35 was less phosphorylated and more readily cleaved by calpain. Thus the developmental alterations in the phosphorylation of p35 may switch the regulation of cdk5 to a different mode. The authors also hypothesize that the age-dependent susceptibility of p35 to calpain-mediated cleavage may be related to age-dependent neuronal cell death in neurodegenerative diseases.

6.3. The relationship between cdk5 and tau phosphorylation

Cdk5 has been hypothesized to induce tau hyperphosphorylation under severe conditions, in particular following calpain-mediated cleavage of the p35 activator protein (Patrick et al. 1999, Ahlijanian et al. 2000, Lee et al. 2000, Nath et al. 2000, Cruz et al. 2003). For this reason, we monitored also the phosphorylation of tau protein in association with cdk5 during experimentally induced death of cultured neurons. Apoptotic neuronal death resulted in generally decreased p35, p25, and cdk5 protein levels and cdk5 activity, which was associated with reduced phosphorylation of the tau protein. However, tau phosphorylation was decreased also during glutamate excitotoxicity, despite of the concomitant calpain-mediated cleavage of p35 to p25, and increased cdk5 activity. In principle, a minor effect of the elevated cdk5 activity on tau phosphorylation could have been masked by other concurrent changes occurring in these conditions. Nevertheless, inhibition of calpain in glutamate-treated neurons had no effect on tau phosphorylation, which suggests that calpain-activated processes, i.e. p35 cleavage to p25, do not contribute to tau phosphorylation. Thus the changes in the regulation of cdk5 during neuronal death do not invariably reflect the phosphorylation status of tau.

Studies regarding tau phosphorylation during neuronal death in culture have produced highly inconsistent results. For instance, treatment of cultured neurons with glutamate agonists or calcium ionophores has resulted in both increased (Mattson 1992, Sindou et al. 1994) and decreased (Davis et al. 1995, Fleming and Johnson 1995, Saito et al. 1995, Adamec et al. 1997, Hull et al. 1999, Lorio et al. 2001, Takashima et al. 2001) phosphorylation of tau. Although these inconsistent results may be partially explained by the different experimental approaches employed, it seems that tau

hyperphosphorylation is not an general event in neuronal death. The molecular mechanisms leading to reduced tau phosphorylation during apoptotic and excitotoxic neuronal death in our study remain elusive. At least during glutamate excitotoxicity, the changes in tau phosphorylation occurred irrespective of the alterations in cdk5. Other investigators have reported that inhibitors of the calcium-dependent protein phosphatase calcineurin prevent dephosphorylation of tau occurring in neurons treated with NMDA, or with a calcium ionophore (Fleming and Johnson 1995, Saito et al. 1995). Activation of calcineurin could have taken place in the conditions used in our study, especially in the neuronal cultures treated with glutamate agonists or calcium ionophores.

Our results showing dephosphorylation of tau protein concomitantly with calpain-mediated cleavage of p35 to p25 are in apparent conflict with the hypothesis that activation of p35 cleavage during neurotoxic conditions would invariably lead to cdk5-mediated tau phosphorylation. That hypothesis is mainly based on the following findings. First, p35 cleavage frequently takes place in various cell culture and *in vivo* conditions involving neuronal death (Kusakawa et al. 2000, Lee et al. 2000, Nath et al. 2000, Nguyen et al. 2001, Bu et al. 2002, Smith et al. 2003). Second, p25 is more efficient than p35 in activating cdk5-mediated tau phosphorylation *in vitro*, in transfected cell cultures, and in transgenic animals (Patrick et al. 1999, Ahlijanian et al. 2000, Hashiguchi et al. 2002, Cruz et al. 2003, Hamdane et al. 2003). However, the evidence suggesting that the calpain-mediated cleavage of the *endogenous* p35 during neuronal death would indeed cause an increase in tau phosphorylation is weak. Our findings indicate that this gap in the current knowledge may be significant. The cellular effects of overexpressed p25, and p25 released from the endogenous p35, could be different for a number of reasons. For instance, overexpressed proteins may be produced in an unphysiologically high amount, in an abnormal subcellular location, or in the absence of other calcium-activated processes. Nevertheless, cleavage of endogenous p35 has been observed in association with increased phosphorylation of tau in some experimental animal models of neurological disorders (Nath et al. 2000, Nguyen et al. 2001, Bu et al. 2002, Smith et al. 2003, Wang et al. 2003). Cdk inhibitors reduce tau phosphorylation in some of these models (Smith et al. 2003, Wen et al. 2004, Zhang et

al. 2004b), however the contribution of cdks other than cdk5 to tau phosphorylation in these conditions is a significant possibility.

The hypothesis regarding the role p25 in cdk5-mediated in tau hyperphosphorylation may require updating. Based on the overexpression models, p25 may indeed possess a greater potential than p35 to induce tau phosphorylation. Nevertheless, the calpain-mediated cleavage of the endogenous p35 as such may not be sufficient to activate tau phosphorylation. It is possible that in neurodegenerative tauopathies there could be some additional factors affecting cdk5, tau, or other cellular elements in such a manner that the calpain-dependent cdk5-mediated effect on tau phosphorylation would be augmented. This kind of additional factors could be involved for instance in the p25-cdk5-mediated phosphorylation of tau detected in neurons exposed to β-amyloid. It was recently reported, that p35 and p25 as such evoke tau phosphorylation in a comparable manner in cultured neurons overexpressing these proteins. However, in the context of amyloid toxicity, there was a striking increase in tau phosphorylation, that could be prevented by specifically inhibiting the function of p25-cdk5 (Zheng et al. 2005). One largely neglected possibility is that cdk5-mediated hyperphosphorylation of tau could take place in a manner completely independent of the calpain-mediated cleavage of cdk5 activators. Further studies will be required to resolve these issues.

7. CONCLUSIONS

The aim of the present study was to investigate the regulation of cdk5, especially during neuronal cell death, and to evaluate the possible association between cdk5 and phosphorylation of the tau protein. The following conclusions can be drawn:

1. Apoptosis induced in rat hippocampal neuronal cultures by different pro-apoptotic stimuli is associated with reduced protein levels of p35, p25, and cdk5, as well as with decreased cdk5 activity and tau phosphorylation.

2. During cell death induced by glutamate, NMDA, and calcium ionophores in cultured neurons, there is prominent calpain-mediated cleavage of p35 to p25. Although this is associated with increased cdk5 activity, at least in the glutamate-treated neurons, the phosphorylation of tau protein is concomitantly reduced.

3. Calpain-mediated cleavage of p35 to p25 can occur downstream of activation of both NMDA and non-NMDA classes of glutamate receptors. The NMDA receptors predominantly mediate the effect of glutamate in the cultured hippocampal neurons. Functional variation in the glutamate receptors related for instance to changes occurring during neuronal maturation, or to differential desensitization properties, may determine the potential of different receptor classes to induce p35 cleavage.

4. A significant proportion of p35 is in a phosphorylated state *in vivo*. Phosphorylation of p35 appears to affect its proteolytic degradation by the proteasome, as well as the calpain-mediated cleavage of p35 to p25. A roscovitine-sensitive kinase, likely cdk5, and another unknown kinase, are able to phosphorylate p35.

In conclusion, this study provides new information on the regulation of cdk5. The findings increase the knowledge required to evaluate the possible role of cdk5 in various neuropathological conditions.

REFERENCES

Aarts MM and Tymianski M. Novel treatment of excitotoxicity: targeted disruption of intracellular signalling from glutamate receptors. Biochem Pharmacol 2003;66:877-886.

Ackerley S, Thornhill P, Grierson AJ, Brownlees J, Anderton BH, Leigh PN, Shaw CE and Miller CC. Neurofilament heavy chain side arm phosphorylation regulates axonal transport of neurofilaments. J Cell Biol 2003;161:489-495.

Adamec E, Mercken M, Beermann ML, Didier M and Nixon RA. Acute rise in the concentration of free cytoplasmic calcium leads to dephosphorylation of the microtubule-associated protein tau. Brain Res 1997;757:93-101.

Ahlijanian MK, Barrezueta NX, Williams RD, Jakowski A, Kowsz KP, McCarthy S, Coskran T, Carlo A, Seymour PA, Burkhardt JE, Nelson RB and McNeish JD. Hyperphosphorylated tau and neurofilament and cytoskeletal disruptions in mice overexpressing human p25, an activator of cdk5. Proc Natl Acad Sci U S A 2000;97:2910-2915.

Akhtar RS, Ness JM and Roth KA. Bcl-2 family regulation of neuronal development and neurodegeneration. Biochim Biophys Acta 2004;1644:189-203.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Walter P. Molecular Biology of the Cell. 4th Edition, New York, NY: Garland Science, 2002.

Alonso A, Zaidi T, Novak M, Grundke-Iqbal I and Iqbal K. Hyperphosphorylation induces self-assembly of tau into tangles of paired helical filaments/straight filaments. Proc Natl Acad Sci U S A 2001;98:6923-6928.

Alvarez A, Munoz JP and Maccioni RB. A Cdk5-p35 stable complex is involved in the beta-amyloid-induced deregulation of Cdk5 activity in hippocampal neurons. Exp Cell Res 2001;264:266-274.

Alvarez A, Toro R, Caceres A and Maccioni RB. Inhibition of tau phosphorylating protein kinase cdk5 prevents beta-amyloid-induced neuronal death. FEBS Lett 1999;459:421-426.

Amin ND, Albers W and Pant HC. Cyclin-dependent kinase 5 (cdk5) activation requires interaction with three domains of p35. J Neurosci Res 2002;67:354-362.

Angelo M, Plattner F, Irvine EE and Giese KP. Improved reversal learning and altered fear conditioning in transgenic mice with regionally restricted p25 expression. Eur J Neurosci 2003;18:423-431.

Arundine M and Tymianski M. Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. Cell Calcium 2003;34:325-337.

Assuncao Guimaraes C and Linden R. Programmed cell deaths. Apoptosis and alternative deathstyles. Eur J Biochem 2004;271:1638-1650.

Avila J, Lucas JJ, Perez M and Hernandez F. Role of tau protein in both physiological and pathological conditions. Physiol Rev 2004;84:361-384.

Bajaj NP, Al-Sarraj ST, Anderson V, Kibble M, Leigh N and Miller CC. Cyclin-dependent kinase-5 is associated with lipofuscin in motor neurones in amyotrophic lateral sclerosis. Neurosci Lett 1998;245:45-48.

Bartus RT, Hayward NJ, Elliott PJ, Sawyer SD, Baker KL, Dean RL, Akiyama A, Straub JA, Harbeson SL and Li Z. Calpain inhibitor AK295 protects neurons from focal brain ischemia. Effects of postocclusion intra-arterial administration. Stroke 1994;25:2265-2270.

Baumann K, Mandelkow EM, Biernat J, Piwnica-Worms H and Mandelkow E. Abnormal Alzheimer-like phosphorylation of tau-protein by cyclin-dependent kinases cdk2 and cdk5. FEBS Lett 1993;336:417-424.

Benn SC and Woolf CJ. Adult neuron survival strategies--slamming on the brakes. Nat Rev Neurosci 2004;5:686-700.

Bezvenyuk Z, Salminen A and Solovyan V. Excision of DNA loop domains as a common step in caspase-dependent and -independent types of neuronal cell death. Brain Res Mol Brain Res 2000;81:191-196.

Bian F, Nath R, Sobocinski G, Booher RN, Lipinski WJ, Callahan MJ, Pack A, Wang KK and Walker LC. Axonopathy, tau abnormalities, and dyskinesia, but no neurofibrillary tangles in p25-transgenic mice. J Comp Neurol 2002;446:257-266.

Bibb JA, Chen J, Taylor JR, Svenningsson P, Nishi A, Snyder GL, Yan Z, Sagawa ZK, Ouimet CC, Nairn AC, Nestler EJ and Greengard P. Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. Nature 2001a;410:376-380.

Bibb JA, Nishi A, O'Callaghan JP, Ule J, Lan M, Snyder GL, Horiuchi A, Saito T, Hisanaga S, Czernik AJ, Nairn AC and Greengard P. Phosphorylation of protein phosphatase inhibitor-1 by Cdk5. J Biol Chem 2001b;276:14490-14497.

Bibb JA, Snyder GL, Nishi A, Yan Z, Meijer L, Fienberg AA, Tsai LH, Kwon YT, Girault JA, Czernik AJ, Huganir RL, Hemmings HC, Jr., Nairn AC and Greengard P. Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signalling in neurons. Nature 1999;402:669-671.

Bonfoco E, Krainc D, Ankarcrona M, Nicotera P and Lipton SA. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci U S A 1995;92:7162-7166.

Bortner CD and Cidlowski JA. Cellular mechanisms for the repression of apoptosis. Annu Rev Pharmacol Toxicol 2002;42:259-281.

Bossy-Wetzel E, Schwarzenbacher R and Lipton SA. Molecular pathways to neurodegeneration. Nat Med 2004;10 Suppl:S2-9.

Braak E, Braak H and Mandelkow EM. A sequence of cytoskeleton changes related to the formation of neurofibrillary tangles and neuropil threads. Acta Neuropathol (Berl) 1994;87:554-567.

Bramlett HM and Dietrich WD. Pathophysiology of cerebral ischemia and brain trauma: similarities and differences. J Cereb Blood Flow Metab 2004;24:133-150.

Brewer GJ, Torricelli JR, Evege EK and Price PJ. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res 1993;35:567-576.

Brion JP and Couck AM. Cortical and brainstem-type Lewy bodies are immunoreactive for the cyclin-dependent kinase 5. Am J Pathol 1995;147:1465-1476.

Brorson JR, Marcuccilli CJ and Miller RJ. Delayed antagonism of calpain reduces excitotoxicity in cultured neurons. Stroke 1995a;26:1259-1266.

Brorson JR, Manzolillo PA, Gibbons SJ and Miller RJ. AMPA receptor desensitization predicts the selective vulnerability of cerebellar Purkinje cells to excitotoxicity. J Neurosci 1995b;15:4515-4524.

Bu B, Li J, Davies P and Vincent I. Deregulation of cdk5, hyperphosphorylation, and cytoskeletal pathology in the Niemann-Pick type C murine model. J Neurosci 2002;22:6515-6525.

Buchan AM, Li H, Cho S and Pulsinelli WA. Blockade of the AMPA receptor prevents CA1 hippocampal injury following severe but transient forebrain ischemia in adult rats. Neurosci Lett 1991;132:255-258.

Buee L, Bussiere T, Buee-Scherrer V, Delacourte A and Hof PR. Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. Brain Res Brain Res Rev 2000;33:95-130.

Cagnoli CM, Kharlamov E, Atabay C, Uz T and Manev H. Apoptosis induced in neuronal cultures by either the phosphatase inhibitor okadaic acid or the kinase inhibitor staurosporine is attenuated by isoquinolinesulfonamides H-7, H-8, and H-9. J Mol Neurosci 1996;7:65-76.

Cai X, Tang D and Xu R. Neuronal cdc-2 like kinase in developing kindling rat hippocampus. Chin Med J (Engl) 2001;114:248-252.

Cai XH, Tomizawa K, Tang D, Lu YF, Moriwaki A, Tokuda M, Nagahata S, Hatase O and Matsui H. Changes in the expression of novel Cdk5 activator messenger RNA (p39nck5ai mRNA) during rat brain development. Neurosci Res 1997;28:355-360.

Canu N, Dus L, Barbato C, Ciotti MT, Brancolini C, Rinaldi AM, Novak M, Cattaneo A, Bradbury A and Calissano P. Tau cleavage and dephosphorylation in cerebellar granule neurons undergoing apoptosis. J Neurosci 1998;18:7061-7074.

Canudas AM, Jorda EG, Verdaguer E, Jimenez A, Sureda FX, Rimbau V, Camins A and Pallas M. Cyclosporin A enhances colchicine-induced apoptosis in rat cerebellar granule neurons. Br J Pharmacol 2004;141:661-669.

Chae T, Kwon YT, Bronson R, Dikkes P, Li E and Tsai LH. Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality. Neuron 1997;18:29-42.

Chen J, Zhang Y, Kelz MB, Steffen C, Ang ES, Zeng L and Nestler EJ. Induction of cyclin-dependent kinase 5 in the hippocampus by chronic electroconvulsive seizures: role of [Delta]FosB. J Neurosci 2000;20:8965-8971.

Cheng C, Fass DM and Reynolds IJ. Emergence of excitotoxicity in cultured forebrain neurons coincides with larger glutamate-stimulated [Ca(2+)](i) increases and NMDA receptor mRNA levels. Brain Res 1999;849:97-108.

Cheng Q, Sasaki Y, Shoji M, Sugiyama Y, Tanaka H, Nakayama T, Mizuki N, Nakamura F, Takei K and Goshima Y. Cdk5/p35 and Rho-kinase mediate ephrin-A5-induced signaling in retinal ganglion cells. Mol Cell Neurosci 2003;24:632-645.

Ching YP, Pang AS, Lam WH, Qi RZ and Wang JH. Identification of a neuronal Cdk5 activator-binding protein as Cdk5 inhibitor. J Biol Chem 2002;277:15237-15240.

Choi DW. Ionic dependence of glutamate neurotoxicity. J Neurosci 1987;7:369-379.

Ciechanover A and Brundin P. The ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg. Neuron 2003;40:427-446.

Clarke PG. Developmental cell death: morphological diversity and multiple mechanisms. Anat Embryol (Berl) 1990;181:195-213.

Courtney MJ and Coffey ET. The mechanism of Ara-C-induced apoptosis of differentiating cerebellar granule neurons. Eur J Neurosci 1999;11:1073-1084.

Creagh EM, Conroy H and Martin SJ. Caspase-activation pathways in apoptosis and immunity. Immunol Rev 2003;193:10-21.

Cruz JC, Tseng HC, Goldman JA, Shih H and Tsai LH. Aberrant Cdk5 activation by p25 triggers pathological events leading to neurodegeneration and neurofibrillary tangles. Neuron 2003;40:471-483.

Curtin JF and Cotter TG. Live and let die: regulatory mechanisms in Fas-mediated apoptosis. Cell Signal 2003;15:983-992.

Danysz W, Parsons CG, Möbius H-J, Stöffler A and Quack G. Neuroprotective and symptomatological action of memantine relevant for Alzheimer's disease - A unified glutamatergic hypothesis on the mechanism of action. Neurotox Res 2000;2:85-97.

Davis DR, Brion JP, Couck AM, Gallo JM, Hanger DP, Ladhani K, Lewis C, Miller CC, Rupniak T, Smith C and Anderton BH. The phosphorylation state of the

microtubule-associated protein tau as affected by glutamate, colchicine and beta-amyloid in primary rat cortical neuronal cultures. Biochem J 1995;309:941-949.

Davis KL, Martin E, Turko IV and Murad F. Novel effects of nitric oxide. Annu Rev Pharmacol Toxicol 2001;41:203-236.

Delalle I, Bhide PG, Caviness VS, Jr. and Tsai LH. Temporal and spatial patterns of expression of p35, a regulatory subunit of cyclin-dependent kinase 5, in the nervous system of the mouse. J Neurocytol 1997;26:283-296.

Desbarats J, Birge RB, Mimouni-Rongy M, Weinstein DE, Palerme JS and Newell MK. Fas engagement induces neurite growth through ERK activation and p35 upregulation. Nat Cell Biol 2003;5:118-125.

Dickson DW. Apoptotic mechanisms in Alzheimer neurofibrillary degeneration: cause or effect? J Clin Invest 2004;114:23-27.

Dingledine R, Borges K, Bowie D and Traynelis SF. The glutamate receptor ion channels. Pharmacol Rev 1999;51:7-61.

Dirnagl U, Iadecola C and Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 1999;22:391-397.

Duchen MR. Roles of mitochondria in health and disease. Diabetes 2004;53 Suppl 1:S96-102.

Earnshaw WC, Martins LM and Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 1999;68:383-424.

Edinger AL and Thompson CB. Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol 2004;16:663-669.

Egana JT, Zambrano C, Nunez MT, Gonzalez-Billault C and Maccioni RB. Iron-induced oxidative stress modify tau phosphorylation patterns in hippocampal cell cultures. Biometals 2003;16:215-223.

Endo S, Zhou X, Connor J, Wang B and Shenolikar S. Multiple structural elements define the specificity of recombinant human inhibitor-1 as a protein phosphatase-1 inhibitor. Biochemistry 1996;35:5220-5228.

Ferrer I and Planas AM. Signaling of cell death and cell survival following focal cerebral ischemia: life and death struggle in the penumbra. J Neuropathol Exp Neurol 2003;62:329-339.

Fischer A, Sananbenesi F, Schrick C, Spiess J and Radulovic J. Cyclin-dependent kinase 5 is required for associative learning. J Neurosci 2002;22:3700-3707.

Fleming LM and Johnson GV. Modulation of the phosphorylation state of tau in situ: the roles of calcium and cyclic AMP. Biochem J 1995;309:41-47.

Fletcher AI, Shuang R, Giovannucci DR, Zhang L, Bittner MA and Stuenkel EL. Regulation of exocytosis by cyclin-dependent kinase 5 via phosphorylation of Munc18. J Biol Chem 1999;274:4027-4035.

Floyd SR, Porro EB, Slepnev VI, Ochoa GC, Tsai LH and De Camilli P. Amphiphysin 1 binds the cyclin-dependent kinase (cdk) 5 regulatory subunit p35 and is phosphorylated by cdk5 and cdc2. J Biol Chem 2001;276:8104-8110.

Frandsen A and Schousboe A. AMPA receptor-mediated neurotoxicity: role of Ca2+ and desensitization. Neurochem Res 2003;28:1495-1499.

Freeman RS, Estus S and Johnson EM, Jr. Analysis of cell cycle-related gene expression in postmitotic neurons: selective induction of Cyclin D1 during programmed cell death. Neuron 1994;12:343-355.

Friedlander RM. Apoptosis and caspases in neurodegenerative diseases. N Engl J Med 2003;348:1365-1375.

Fu WY, Wang JH and Ip NY. Expression of Cdk5 and its activators in NT2 cells during neuronal differentiation. J Neurochem 2002;81:646-654.

Gilmore EC, Ohshima T, Goffinet AM, Kulkarni AB and Herrup K. Cyclin-dependent kinase 5-deficient mice demonstrate novel developmental arrest in cerebral cortex. J Neurosci 1998;18:6370-6377.

Goll DE, Thompson VF, Li H, Wei W and Cong J. The calpain system. Physiol Rev 2003;83:731-801.

Gong X, Tang X, Wiedmann M, Wang X, Peng J, Zheng D, Blair LA, Marshall J and Mao Z. Cdk5-mediated inhibition of the protective effects of transcription factor MEF2 in neurotoxicity-induced apoptosis. Neuron 2003;38:33-46.

Green DR and Kroemer G. The pathophysiology of mitochondrial cell death. Science 2004;305:626-629.

Gulbins E, Dreschers S and Bock J. Role of mitochondria in apoptosis. Exp Physiol 2003;88:85-90.

Gunter TE, Yule DI, Gunter KK, Eliseev RA and Salter JD. Calcium and mitochondria. FEBS Lett 2004;567:96-102.

Gupta A and Tsai LH. Cyclin-dependent kinase 5 and neuronal migration in the neocortex. Neurosignals 2003;12:173-179.

Hajnoczky G, Davies E and Madesh M. Calcium signaling and apoptosis. Biochem Biophys Res Commun 2003;304:445-454.

Hamdane M, Sambo AV, Delobel P, Begard S, Violleau A, Delacourte A, Bertrand P, Benavides J and Buee L. Mitotic-like tau phosphorylation by p25-Cdk5 kinase complex. J Biol Chem 2003;278:34026-34034.

Haque N, Gong CX, Sengupta A, Iqbal K and Grundke-Iqbal I. Regulation of microtubule-associated proteins, protein kinases and protein phosphatases during differentiation of SY5Y cells. Brain Res Mol Brain Res 2004;129:163-170.

Harada T, Morooka T, Ogawa S and Nishida E. ERK induces p35, a neuron-specific activator of Cdk5, through induction of Egr1. Nat Cell Biol 2001;3:453-459.

Hashiguchi M, Saito T, Hisanaga S and Hashiguchi T. Truncation of CDK5 activator p35 induces intensive phosphorylation of Ser202/Thr205 of human tau. J Biol Chem 2002;277:44525-44530.

Hayashi T, Warita H, Abe K and Itoyama Y. Expression of cyclin-dependent kinase 5 and its activator p35 in rat brain after middle cerebral artery occlusion. Neurosci Lett 1999;265:37-40.

Hayes TE, Valtz NL and McKay RD. Downregulation of CDC2 upon terminal differentiation of neurons. New Biol 1991;3:259-269.

Hellmich MR, Pant HC, Wada E and Battey JF. Neuronal cdc2-like kinase: a cdc2-related protein kinase with predominantly neuronal expression. Proc Natl Acad Sci U S A 1992;89:10867-10871.

Hemmings HC, Jr., Greengard P, Tung HY and Cohen P. DARPP-32, a dopamine-regulated neuronal phosphoprotein, is a potent inhibitor of protein phosphatase-1. Nature 1984;310:503-505.

Henchcliffe C and Burke RE. Increased expression of cyclin-dependent kinase 5 in induced apoptotic neuron death in rat substantia nigra. Neurosci Lett 1997;230:41-44.

Hetman M and Gozdz A. Role of extracellular signal regulated kinases 1 and 2 in neuronal survival. Eur J Biochem 2004;271:2050-2055.

Honjyo Y, Kawamoto Y, Nakamura S, Nakano S and Akiguchi I. Immunohistochemical localization of CDK5 activator p39 in the rat brain. Neuroreport 1999;10:3375-3379.

Hull M, Eistetter J, Fiebich BL and Bauer J. Glutamate but not interleukin-6 influences the phosphorylation of tau in primary rat hippocampal neurons. Neurosci Lett 1999;261:33-36.

Humbert S, Dhavan R and Tsai L. p39 activates cdk5 in neurons, and is associated with the actin cytoskeleton. J Cell Sci 2000;113:975-983.

Iijima K, Ando K, Takeda S, Satoh Y, Seki T, Itohara S, Greengard P, Kirino Y, Nairn AC and Suzuki T. Neuron-specific phosphorylation of Alzheimer's beta-amyloid precursor protein by cyclin-dependent kinase 5. J Neurochem 2000;75:1085-1091.

Ino H and Chiba T. Intracellular localization of cyclin-dependent kinase 5 (CDK5) in mouse neuron: CDK5 is located in both nucleus and cytoplasm. Brain Res 1996;732:179-185.

Ishiguro K, Takamatsu M, Tomizawa K, Omori A, Takahashi M, Arioka M, Uchida T and Imahori K. Tau protein kinase I converts normal tau protein into A68-like component of paired helical filaments. J Biol Chem 1992;267:10897-10901.

Jeong YG, Rosales JL, Marzban H, Sillitoe RV, Park DG, Hawkes R and Lee KY. The cyclin-dependent kinase 5 activator, p39, is expressed in stripes in the mouse cerebellum. Neuroscience 2003;118:323-334.

Jorda EG, Verdaguer E, Canudas AM, Jimenez A, Bruna A, Caelles C, Bravo R, Escubedo E, Pubill D, Camarasa J, Pallas M and Camins A. Neuroprotective action of flavopiridol, a cyclin-dependent kinase inhibitor, in colchicine-induced apoptosis. Neuropharmacology 2003;45:672-683.

Kaminska B, Figiel I, Pyrzynska B, Czajkowski R and Mosieniak G. Treatment of hippocampal neurons with cyclosporin A results in calcium overload and apoptosis which are independent on NMDA receptor activation. Br J Pharmacol 2001;133:997-1004.

Kandel ER, Schwartz JH and Jessel TM. Essentials of Neural Science and Behavior. Appleton and Lange, 1995.

Kerr JF, Wyllie AH and Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239-257.

Kesavapany S, Lau KF, McLoughlin DM, Brownlees J, Ackerley S, Leigh PN, Shaw CE and Miller CC. p35/cdk5 binds and phosphorylates beta-catenin and regulates beta-catenin/presenilin-1 interaction. Eur J Neurosci 2001;13:241-247.

Kesavapany S, Amin N, Zheng YL, Nijhara R, Jaffe H, Sihag R, Gutkind JS, Takahashi S, Kulkarni A, Grant P and Pant HC. p35/cyclin-dependent kinase 5 phosphorylation of ras guanine nucleotide releasing factor 2 (RasGRF2) mediates Rac-dependent Extracellular Signal-regulated kinase 1/2 activity, altering RasGRF2 and microtubule-associated protein 1b distribution in neurons. J Neurosci 2004;24:4421-4431.

Kim JE, Han BS, Choi WS, Eom DS, Lee EH, Oh TH, Markelonis GJ, Saido TC, Lee GE, Chung IK and Oh YJ. Temporospatial sequence of cellular events associated with etoposide-induced neuronal cell death: role of antiapoptotic protein Bcl-X(L). J Neurosci Res 2001;66:1074-1082.

Ko HW, Han KS, Kim EY, Ryu BR, Yoon WJ, Jung YK, Kim SU and Gwag BJ. Synergetic activation of p38 mitogen-activated protein kinase and caspase-3-like proteases for execution of calyculin A-induced apoptosis but not N-methyl-d-aspartate-induced necrosis in mouse cortical neurons. J Neurochem 2000;74:2455-2461.

Ko J, Humbert S, Bronson RT, Takahashi S, Kulkarni AB, Li E and Tsai LH. p35 and p39 are essential for cyclin-dependent kinase 5 function during neurodevelopment. J Neurosci 2001;21:6758-6771.

Kobayashi S, Ishiguro K, Omori A, Takamatsu M, Arioka M, Imahori K and Uchida T. A cdc2-related kinase PSSALRE/cdk5 is homologous with the 30 kDa subunit of tau

protein kinase II, a proline-directed protein kinase associated with microtubule. FEBS Lett 1993;335:171-175.

Krishnamurthy PK, Mays JL, Bijur GN and Johnson GV. Transient oxidative stress in SH-SY5Y human neuroblastoma cells results in caspase dependent and independent cell death and tau proteolysis. J Neurosci Res 2000;61:515-523.

Kruman I, Bruce-Keller AJ, Bredesen D, Waeg G and Mattson MP. Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. J Neurosci 1997;17:5089-5100.

Kusakawa G, Saito T, Onuki R, Ishiguro K, Kishimoto T and Hisanaga S. Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25. J Biol Chem 2000;275:17166-17172.

Kwon YT, Tsai LH and Crandall JE. Callosal axon guidance defects in p35(-/-) mice. J Comp Neurol 1999;415:218-229.

Kwon YT, Gupta A, Zhou Y, Nikolic M and Tsai LH. Regulation of N-cadherin-mediated adhesion by the p35-Cdk5 kinase. Curr Biol 2000;10:363-372.

Lankiewicz S, Marc Luetjens C, Truc Bui N, Krohn AJ, Poppe M, Cole GM, Saido TC and Prehn JH. Activation of calpain I converts excitotoxic neuron death into a caspase-independent cell death. J Biol Chem 2000;275:17064-17071.

Lau KF, Howlett DR, Kesavapany S, Standen CL, Dingwall C, McLoughlin DM and Miller CC. Cyclin-dependent kinase-5/p35 phosphorylates Presenilin 1 to regulate carboxy-terminal fragment stability. Mol Cell Neurosci 2002;20:13-20.

Ledda F, Paratcha G and Ibanez CF. Target-derived GFRalpha1 as an attractive guidance signal for developing sensory and sympathetic axons via activation of Cdk5. Neuron 2002;36:387-401.

Lee JH and Kim KT. Induction of cyclin-dependent kinase 5 and its activator p35 through the extracellular-signal-regulated kinase and protein kinase A pathways during retinoic-acid mediated neuronal differentiation in human neuroblastoma SK-N-BE(2)C cells. J Neurochem 2004;91:634-647.

Lee MH, Nikolic M, Baptista CA, Lai E, Tsai LH and Massague J. The brain-specific activator p35 allows Cdk5 to escape inhibition by p27Kip1 in neurons. Proc Natl Acad Sci U S A 1996;93:3259-3263.

Lee MS, Kwon YT, Li M, Peng J, Friedlander RM and Tsai LH. Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature 2000;405:360-364.

Lee SY, Wenk MR, Kim Y, Nairn AC and De Camilli P. Regulation of synaptojanin 1 by cyclin-dependent kinase 5 at synapses. Proc Natl Acad Sci U S A 2004;101:546-551.

Lee VM, Goedert M and Trojanowski JQ. Neurodegenerative tauopathies. Annu Rev Neurosci 2001;24:1121-1159.

Leist M and Nicotera P. Calcium and Cell Death, in Koliatsos VE and Ratan RR, eds, Cell Death and Diseases of the Nervous System. Totowa, NJ: Humana Press Inc., 1999, pp. 69-90.

Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, Crowe RA, Cascio WE, Bradham CA, Brenner DA and Herman B. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim Biophys Acta 1998;1366:177-196.

Le-Niculescu H, Bonfoco E, Kasuya Y, Claret FX, Green DR and Karin M. Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. Mol Cell Biol 1999;19:751-763.

Lew J, Huang QQ, Qi Z, Winkfein RJ, Aebersold R, Hunt T and Wang JH. A brain-specific activator of cyclin-dependent kinase 5. Nature 1994;371:423-426.

Li BS, Zhang L, Takahashi S, Ma W, Jaffe H, Kulkarni AB and Pant HC. Cyclin-dependent kinase 5 prevents neuronal apoptosis by negative regulation of c-Jun N-terminal kinase 3. EMBO J 2002;21:324-333.

Li BS, Ma W, Jaffe H, Zheng Y, Takahashi S, Zhang L, Kulkarni AB and Pant HC. Cyclin-dependent kinase-5 is involved in neuregulin-dependent activation of phosphatidylinositol 3-kinase and Akt activity mediating neuronal survival. J Biol Chem 2003;278:35702-35709.

Li BS, Sun MK, Zhang L, Takahashi S, Ma W, Vinade L, Kulkarni AB, Brady RO and Pant HC. Regulation of NMDA receptors by cyclin-dependent kinase-5. Proc Natl Acad Sci U S A 2001;98:12742-12747.

Li Y, Powers C, Jiang N and Chopp M. Intact, injured, necrotic and apoptotic cells after focal cerebral ischemia in the rat. J Neurol Sci 1998;156:119-132.

Lim AC, Qu D and Qi RZ. Protein-protein interactions in Cdk5 regulation and function. Neurosignals 2003;12:230-238.

Lim AC, Hou Z, Goh CP and Qi RZ. Protein kinase CK2 is an inhibitor of the neuronal Cdk5 kinase. J Biol Chem 2004;279:46668-46673.

Liou AK, Clark RS, Henshall DC, Yin XM and Chen J. To die or not to die for neurons in ischemia, traumatic brain injury and epilepsy: a review on the stress-activated signaling pathways and apoptotic pathways. Prog Neurobiol 2003;69:103-142.

Lipton SA and Rosenberg PA. Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med 1994;330:613-622.

Liu F, Virshup DM, Nairn AC and Greengard P. Mechanism of regulation of casein kinase I activity by group I metabotropic glutamate receptors. J Biol Chem 2002;277:45393-45399.

Liu F, Su Y, Li B, Zhou Y, Ryder J, Gonzalez-DeWhitt P, May PC and Ni B. Regulation of amyloid precursor protein (APP) phosphorylation and processing by p35/Cdk5 and p25/Cdk5. FEBS Lett 2003;547:193-196.

Liu F, Ma XH, Ule J, Bibb JA, Nishi A, DeMaggio AJ, Yan Z, Nairn AC and Greengard P. Regulation of cyclin-dependent kinase 5 and casein kinase 1 by metabotropic glutamate receptors. Proc Natl Acad Sci U S A 2001;98:11062-11068.

Liu S, Lau L, Wei J, Zhu D, Zou S, Sun HS, Fu Y, Liu F and Lu Y. Expression of Ca(2+)-permeable AMPA receptor channels primes cell death in transient forebrain ischemia. Neuron 2004;43:43-55.

Lorio G, Avila J and Diaz-Nido J. Modifications of tau protein during neuronal cell death. J Alzheimers Dis 2001;3:563-575.

Lossi L and Merighi A. In vivo cellular and molecular mechanisms of neuronal apoptosis in the mammalian CNS. Prog Neurobiol 2003;69:287-312.

Lund ET, McKenna R, Evans DB, Sharma SK and Mathews WR. Characterization of the in vitro phosphorylation of human tau by tau protein kinase II (cdk5/p20) using mass spectrometry. J Neurochem 2001;76:1221-1232.

Marino G and Lopez-Otin C. Autophagy: molecular mechanisms, physiological functions and relevance in human pathology. Cell Mol Life Sci 2004;61:1439-1454.

Matsushita M, Tomizawa K, Lu YF, Moriwaki A, Tokuda M, Itano T, Wang JH, Hatase O and Matsui H. Distinct cellular compartment of cyclin-dependent kinase 5 (Cdk5) and neuron-specific Cdk5 activator protein (p35nck5a) in the developing rat cerebellum. Brain Res 1996;734:319-322.

Matsuura I and Wang JH. Demonstration of cyclin-dependent kinase inhibitory serine/threonine kinase in bovine thymus. J Biol Chem 1996;271:5443-5450.

Mattson MP. Effects of microtubule stabilization and destabilization on tau immunoreactivity in cultured hippocampal neurons. Brain Res 1992;582:107-118.

Mattson MP. Pathways towards and away from Alzheimer's disease. Nature 2004;430:631-639.

McDonald JW, Goldberg MP, Gwag BJ, Chi SI and Choi DW. Cyclosporine induces neuronal apoptosis and selective oligodendrocyte death in cortical cultures. Ann Neurol 1996;40:750-758.

McGeer EG and McGeer PL. Inflammatory processes in Alzheimer's disease. Prog Neuropsychopharmacol Biol Psychiatry 2003;27:741-749.

Meijer L, Borgne A, Mulner O, Chong JP, Blow JJ, Inagaki N, Inagaki M, Delcros JG and Moulinoux JP. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. Eur J Biochem 1997;243:527-536.

Meyerson M, Enders GH, Wu CL, Su LK, Gorka C, Nelson C, Harlow E and Tsai LH. A family of human cdc2-related protein kinases. EMBO J 1992;11:2909-2917.

Moorthamer M and Chaudhuri B. Identification of ribosomal protein L34 as a novel Cdk5 inhibitor. Biochem Biophys Res Commun 1999;255:631-638.

Moorthamer M, Zumstein-Mecker S and Chaudhuri B. DNA binding protein dbpA binds Cdk5 and inhibits its activity. FEBS Lett 1999;446:343-350.

Morabito MA, Sheng M and Tsai LH. Cyclin-dependent kinase 5 phosphorylates the N-terminal domain of the postsynaptic density protein PSD-95 in neurons. J Neurosci 2004;24:865-876.

Nakajima M, Kashiwagi K, Ohta J, Furukawa S, Hayashi K, Kawashima T and Hayashi Y. Etoposide induces programmed death in neurons cultured from the fetal rat central nervous system. Brain Res 1994;641:350-352.

Nakamura S, Kawamoto Y, Nakano S, Akiguchi I and Kimura J. p35nck5a and cyclin-dependent kinase 5 colocalize in Lewy bodies of brains with Parkinson's disease. Acta Neuropathol (Berl) 1997a;94:153-157.

Nakamura S, Kawamoto Y, Nakano S, Ikemoto A, Akiguchi I and Kimura J. Cyclin-dependent kinase 5 in Lewy body-like inclusions in anterior horn cells of a patient with sporadic amyotrophic lateral sclerosis. Neurology 1997b;48:267-270.

Nakanishi H. Neuronal and microglial cathepsins in aging and age-related diseases. Ageing Res Rev 2003;2:367-381.

Namgung U, Choi BH, Park S, Lee JU, Seo HS, Suh BC and Kim KT. Activation of cyclin-dependent kinase 5 is involved in axonal regeneration. Mol Cell Neurosci 2004;25:422-432.

Nath R, Davis M, Probert AW, Kupina NC, Ren X, Schielke GP and Wang KK. Processing of cdk5 activator p35 to its truncated form (p25) by calpain in acutely injured neuronal cells. Biochem Biophys Res Commun 2000;274:16-21.

Neystat M, Rzhetskaya M, Oo TF, Kholodilov N, Yarygina O, Wilson A, El-Khodor BF and Burke RE. Expression of cyclin-dependent kinase 5 and its activator p35 in models of induced apoptotic death in neurons of the substantia nigra in vivo. J Neurochem 2001;77:1611-1625.

Nguyen MD, Lariviere RC and Julien JP. Deregulation of Cdk5 in a mouse model of ALS: toxicity alleviated by perikaryal neurofilament inclusions. Neuron 2001;30:135-147.

Niethammer M, Smith DS, Ayala R, Peng J, Ko J, Lee MS, Morabito M and Tsai LH. NUDEL is a novel Cdk5 substrate that associates with LIS1 and cytoplasmic dynein. Neuron 2000;28:697-711.

Nikolic M. The role of Rho GTPases and associated kinases in regulating neurite outgrowth. Int J Biochem Cell Biol 2002;34:731-745.

Nikolic M, Dudek H, Kwon YT, Ramos YF and Tsai LH. The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation. Genes Dev 1996;10:816-825.

Nikolic M, Chou MM, Lu W, Mayer BJ and Tsai LH. The p35/Cdk5 kinase is a neuron-specific Rac effector that inhibits Pak1 activity. Nature 1998;395:194-198.

Nishi A, Bibb JA, Snyder GL, Higashi H, Nairn AC and Greengard P. Amplification of dopaminergic signaling by a positive feedback loop. Proc Natl Acad Sci U S A 2000;97:12840-12845.

Nitatori T, Sato N, Waguri S, Karasawa Y, Araki H, Shibanai K, Kominami E and Uchiyama Y. Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. J Neurosci 1995;15:1001-1011.

Nixon RA, Wegiel J, Kumar A, Yu WH, Peterhoff C, Cataldo A and Cuervo AM. Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study. J Neuropathol Exp Neurol 2005;64:113-122.

Noble W, Olm V, Takata K, Casey E, Mary O, Meyerson J, Gaynor K, LaFrancois J, Wang L, Kondo T, Davies P, Burns M, Veeranna, Nixon R, Dickson D, Matsuoka Y, Ahlijanian M, Lau LF and Duff K. Cdk5 is a key factor in tau aggregation and tangle formation in vivo. Neuron 2003;38:555-565.

Obaya AJ and Sedivy JM. Regulation of cyclin-Cdk activity in mammalian cells. Cell Mol Life Sci 2002;59:126-142.

Odle TG. Alzheimer disease and other dementias. Radiol Technol 2003;75:111-135.

Ohshima T, Ward JM, Huh CG, Longenecker G, Veeranna, Pant HC, Brady RO, Martin LJ and Kulkarni AB. Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. Proc Natl Acad Sci U S A 1996;93:11173-11178.

Orrenius S, Zhivotovsky B and Nicotera P. Regulation of cell death: the calcium-apoptosis link. Nat Rev Mol Cell Biol 2003;4:552-565.

Otth C, Concha, II, Arendt T, Stieler J, Schliebs R, Gonzalez-Billault C and Maccioni RB. AbetaPP induces cdk5-dependent tau hyperphosphorylation in transgenic mice Tg2576. J Alzheimers Dis 2002;4:417-430.

Ozyurt E, Graham DI, Woodruff GN and McCulloch J. Protective effect of the glutamate antagonist, MK-801 in focal cerebral ischemia in the cat. J Cereb Blood Flow Metab 1988;8:138-143.

Paglini G, Pigino G, Kunda P, Morfini G, Maccioni R, Quiroga S, Ferreira A and Caceres A. Evidence for the participation of the neuron-specific CDK5 activator P35 during laminin-enhanced axonal growth. J Neurosci 1998;18:9858-9869.

Patrick GN, Zhou P, Kwon YT, Howley PM and Tsai LH. p35, the neuronal-specific activator of cyclin-dependent kinase 5 (Cdk5) is degraded by the ubiquitin-proteasome pathway. J Biol Chem 1998;273:24057-24064.

Patrick GN, Zukerberg L, Nikolic M, de la Monte S, Dikkes P and Tsai LH. Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature 1999;402:615-622.

Patzke H and Tsai LH. Calpain-mediated cleavage of the cyclin-dependent kinase-5 activator p39 to p29. J Biol Chem 2002;277:8054-8060.

Pei JJ, Grundke-Iqbal I, Iqbal K, Bogdanovic N, Winblad B and Cowburn RF. Accumulation of cyclin-dependent kinase 5 (cdk5) in neurons with early stages of Alzheimer's disease neurofibrillary degeneration. Brain Res 1998;797:267-277.

Pellegrini-Giampietro DE, Zukin RS, Bennett MV, Cho S and Pulsinelli WA. Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. Proc Natl Acad Sci U S A 1992;89:10499-10503.

Peterson C, Neal JH and Cotman CW. Development of N-methyl-D-aspartate excitotoxicity in cultured hippocampal neurons. Brain Res Dev Brain Res 1989;48:187-195.

Pigino G, Paglini G, Ulloa L, Avila J and Caceres A. Analysis of the expression, distribution and function of cyclin dependent kinase 5 (cdk5) in developing cerebellar macroneurons. J Cell Sci 1997;110:257-270.

Poon RY, Lew J and Hunter T. Identification of functional domains in the neuronal Cdk5 activator protein. J Biol Chem 1997;272:5703-5708.

Proskuryakov SY, Konoplyannikov AG and Gabai VL. Necrosis: a specific form of programmed cell death? Exp Cell Res 2003;283:1-16.

Qi Z, Huang QQ, Lee KY, Lew J and Wang JH. Reconstitution of neuronal Cdc2-like kinase from bacteria-expressed Cdk5 and an active fragment of the brain-specific activator. Kinase activation in the absence of Cdk5 phosphorylation. J Biol Chem 1995;270:10847-10854.

Qu D, Li Q, Lim HY, Cheung NS, Li R, Wang JH and Qi RZ. The protein SET binds the neuronal Cdk5 activator p35nck5a and modulates Cdk5/p35nck5a activity. J Biol Chem 2002;277:7324-7332.

Rabacchi SA, Friedman WJ, Shelanski ML and Troy CM. Divergence of the apoptotic pathways induced by 4-hydroxynonenal and amyloid beta-protein. Neurobiol Aging 2004;25:1057-1066.

Raina AK, Hochman A, Ickes H, Zhu X, Ogawa O, Cash AD, Shimohama S, Perry G and Smith MA. Apoptotic promoters and inhibitors in Alzheimer's disease: Who wins out? Prog Neuropsychopharmacol Biol Psychiatry 2003;27:251-254.

Rami A. Ischemic neuronal death in the rat hippocampus: the calpain-calpastatin-caspase hypothesis. Neurobiol Dis 2003;13:75-88.

Raoul C, Henderson CE and Pettmann B. Programmed cell death of embryonic motoneurons triggered through the Fas death receptor. J Cell Biol 1999;147:1049-1062.

Rashid T, Banerjee M and Nikolic M. Phosphorylation of Pak1 by the p35/Cdk5 kinase affects neuronal morphology. J Biol Chem 2001;276:49043-49052.

Rosales J, Han B and Lee KY. Cdk7 functions as a cdk5 activating kinase in brain. Cell Physiol Biochem 2003;13:285-296.

Ross CA and Poirier MA. Protein aggregation and neurodegenerative disease. Nat Med 2004;10 Suppl:S10-17.

Ryder J, Su Y, Liu F, Li B, Zhou Y and Ni B. Divergent roles of GSK3 and CDK5 in APP processing. Biochem Biophys Res Commun 2003;312:922-929.

Saito T, Ishiguro K, Uchida T, Miyamoto E, Kishimoto T and Hisanaga S. In situ dephosphorylation of tau by protein phosphatase 2A and 2B in fetal rat primary cultured neurons. FEBS Lett 1995;376:238-242.

Saito T, Ishiguro K, Onuki R, Nagai Y, Kishimoto T and Hisanaga S. Okadaic acid-stimulated degradation of p35, an activator of CDK5, by proteasome in cultured neurons. Biochem Biophys Res Commun 1998;252:775-778.

Saito T, Onuki R, Fujita Y, Kusakawa G, Ishiguro K, Bibb JA, Kishimoto T and Hisanaga S. Developmental regulation of the proteolysis of the p35 cyclin-dependent kinase 5 activator by phosphorylation. J Neurosci 2003;23:1189-1197.

Sasaki S, Shionoya A, Ishida M, Gambello MJ, Yingling J, Wynshaw-Boris A and Hirotsune S. A LIS1/NUDEL/cytoplasmic dynein heavy chain complex in the developing and adult nervous system. Neuron 2000;28:681-696.

Sasaki Y, Cheng C, Uchida Y, Nakajima O, Ohshima T, Yagi T, Taniguchi M, Nakayama T, Kishida R, Kudo Y, Ohno S, Nakamura F and Goshima Y. Fyn and Cdk5 mediate semaphorin-3A signaling, which is involved in regulation of dendrite orientation in cerebral cortex. Neuron 2002;35:907-920.

Sattler R, Charlton MP, Hafner M and Tymianski M. Distinct influx pathways, not calcium load, determine neuronal vulnerability to calcium neurotoxicity. J Neurochem 1998;71:2349-2364.

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH and Peter ME. Two CD95 (APO-1/Fas) signaling pathways. Embo J 1998;17:1675-1687.

Schneider A, Araujo GW, Trajkovic K, Herrmann MM, Merkler D, Mandelkow EM, Weissert R and Simons M. Hyperphosphorylation and aggregation of tau in experimental autoimmune encephalomyelitis. J Biol Chem 2004;279:55833-55839.

Schuman EM and Murase S. Cadherins and synaptic plasticity: activity-dependent cyclin-dependent kinase 5 regulation of synaptic beta-catenin-cadherin interactions. Philos Trans R Soc Lond B Biol Sci 2003;358:749-756.

Sharma M, Sharma P and Pant HC. CDK-5-mediated neurofilament phosphorylation in SHSY5Y human neuroblastoma cells. J Neurochem 1999a;73:79-86.

Sharma P, Sharma M, Amin ND, Albers RW and Pant HC. Regulation of cyclin-dependent kinase 5 catalytic activity by phosphorylation. Proc Natl Acad Sci U S A 1999b;96:11156-11160.

Sharma P, Veeranna, Sharma M, Amin ND, Sihag RK, Grant P, Ahn N, Kulkarni AB and Pant HC. Phosphorylation of MEK1 by cdk5/p35 down-regulates the mitogen-activated protein kinase pathway. J Biol Chem 2002;277:528-534.

Shastry BS. Neurodegenerative disorders of protein aggregation. Neurochem Int 2003;43:1-7.

Shea TB, Yabe JT, Ortiz D, Pimenta A, Loomis P, Goldman RD, Amin N and Pant HC. Cdk5 regulates axonal transport and phosphorylation of neurofilaments in cultured neurons. J Cell Sci 2004;117:933-941.

Shintani T and Klionsky DJ. Autophagy in health and disease: a double-edged sword. Science 2004;306:990-995.

Shirvan A, Ziv I, Zilkha-Falb R, Machlyn T, Barzilai A and Melamed E. Expression of cell cycle-related genes during neuronal apoptosis: is there a distinct pattern? Neurochem Res 1998;23:767-777.

Shuang R, Zhang L, Fletcher A, Groblewski GE, Pevsner J and Stuenkel EL. Regulation of Munc-18/syntaxin 1A interaction by cyclin-dependent kinase 5 in nerve endings. J Biol Chem 1998;273:4957-4966.

Siegel GJ, Agranoff BW, Albers RW, Fisher SK and Uhler MD. Basic Neurochemistry. 6th Edition, Philadelphia, PA: Lippincott, Williams & Wilkins, 1999.

Simon RP, Swan JH, Griffiths T and Meldrum BS. Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. Science 1984;226:850-852.

Sindou P, Lesort M, Couratier P, Yardin C, Esclaire F and Hugon J. Glutamate increases tau phosphorylation in primary neuronal cultures from fetal rat cerebral cortex. Brain Res 1994;646:124-128.

Smith PD, Crocker SJ, Jackson-Lewis V, Jordan-Sciutto KL, Hayley S, Mount MP, O'Hare MJ, Callaghan S, Slack RS, Przedborski S, Anisman H and Park DS. Cyclin-

dependent kinase 5 is a mediator of dopaminergic neuron loss in a mouse model of Parkinson's disease. Proc Natl Acad Sci U S A 2003;100:13650-13655.

Sobue K, Agarwal-Mawal A, Li W, Sun W, Miura Y and Paudel HK. Interaction of neuronal Cdc2-like protein kinase with microtubule-associated protein tau. J Biol Chem 2000;275:16673-16680.

Sprick MR and Walczak H. The interplay between the Bcl-2 family and death receptor-mediated apoptosis. Biochim Biophys Acta 2004;1644:125-132.

Stadelmann C, Bruck W, Bancher C, Jellinger K and Lassmann H. Alzheimer disease: DNA fragmentation indicates increased neuronal vulnerability, but not apoptosis. J Neuropathol Exp Neurol 1998;57:456-464.

Stadelmann C, Deckwerth TL, Srinivasan A, Bancher C, Bruck W, Jellinger K and Lassmann H. Activation of caspase-3 in single neurons and autophagic granules of granulovacuolar degeneration in Alzheimer's disease. Evidence for apoptotic cell death. Am J Pathol 1999;155:1459-1466.

Sugiyama H, Ito I and Hirono C. A new type of glutamate receptor linked to inositol phospholipid metabolism. Nature 1987;325:531-533.

Sun D, Leung CL and Liem RK. Phosphorylation of the high molecular weight neurofilament protein (NF-H) by Cdk5 and p35. J Biol Chem 1996;271:14245-14251.

Syntichaki P and Tavernarakis N. The biochemistry of neuronal necrosis: rogue biology? Nat Rev Neurosci 2003;4:672-684.

Takashima A, Murayama M, Yasutake K, Takahashi H, Yokoyama M and Ishiguro K. Involvement of cyclin dependent kinase5 activator p25 on tau phosphorylation in mouse brain. Neurosci Lett 2001;306:37-40.

Tan TC, Valova VA, Malladi CS, Graham ME, Berven LA, Jupp OJ, Hansra G, McClure SJ, Sarcevic B, Boadle RA, Larsen MR, Cousin MA and Robinson PJ. Cdk5 is essential for synaptic vesicle endocytosis. Nat Cell Biol 2003;5:701-710.

Tanaka H, Grooms SY, Bennett MV and Zukin RS. The AMPAR subunit GluR2: still front and center-stage. Brain Res 2000;886:190-207.

Tanaka T, Serneo FF, Tseng HC, Kulkarni AB, Tsai LH and Gleeson JG. Cdk5 phosphorylation of doublecortin ser297 regulates its effect on neuronal migration. Neuron 2004;41:215-227.

Tandon A, Yu H, Wang L, Rogaeva E, Sato C, Chishti MA, Kawarai T, Hasegawa H, Chen F, Davies P, Fraser PE, Westaway D and St George-Hyslop PH. Brain levels of CDK5 activator p25 are not increased in Alzheimer's or other neurodegenerative diseases with neurofibrillary tangles. J Neurochem 2003;86:572-581.

Tang D, Yeung J, Lee KY, Matsushita M, Matsui H, Tomizawa K, Hatase O and Wang JH. An isoform of the neuronal cyclin-dependent kinase 5 (Cdk5) activator. J Biol Chem 1995;270:26897-26903.

Taniguchi S, Fujita Y, Hayashi S, Kakita A, Takahashi H, Murayama S, Saido TC, Hisanaga S, Iwatsubo T and Hasegawa M. Calpain-mediated degradation of p35 to p25 in postmortem human and rat brains. FEBS Lett 2001;489:46-50.

Tarricone C, Dhavan R, Peng J, Areces LB, Tsai LH and Musacchio A. Structure and regulation of the CDK5-p25(nck5a) complex. Mol Cell 2001;8:657-669.

Taru H, Yoshikawa K and Suzuki T. Suppression of the caspase cleavage of beta-amyloid precursor protein by its cytoplasmic phosphorylation. FEBS Lett 2004;567:248-252.

Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, Hansen LA and Katzman R. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann Neurol 1991;30:572-580.

Tokuoka H, Saito T, Yorifuji H, Wei F, Kishimoto T and Hisanaga S. Brain-derived neurotrophic factor-induced phosphorylation of neurofilament-H subunit in primary cultures of embryo rat cortical neurons. J Cell Sci 2000;113:1059-1068.

Tomidokoro Y, Ishiguro K, Harigaya Y, Matsubara E, Ikeda M, Park JM, Yasutake K, Kawarabayashi T, Okamoto K and Shoji M. Abeta amyloidosis induces the initial stage of tau accumulation in APP(Sw) mice. Neurosci Lett 2001;299:169-172.

Tomizawa K, Cai XH, Moriwaki A, Matsushita M and Matsui H. Involvement of cyclin-dependent kinase 5/p35(nck5a) in the synaptic reorganization of rat hippocampus during kindling progression. Jpn J Physiol 2000;50:525-532.

Tomizawa K, Ohta J, Matsushita M, Moriwaki A, Li ST, Takei K and Matsui H. Cdk5/p35 regulates neurotransmitter release through phosphorylation and downregulation of P/Q-type voltage-dependent calcium channel activity. J Neurosci 2002;22:2590-2597.

Tomizawa K, Matsui H, Matsushita M, Lew J, Tokuda M, Itano T, Konishi R, Wang JH and Hatase O. Localization and developmental changes in the neuron-specific cyclin-dependent kinase 5 activator (p35nck5a) in the rat brain. Neuroscience 1996;74:519-529.

Tomizawa K, Sunada S, Lu YF, Oda Y, Kinuta M, Ohshima T, Saito T, Wei FY, Matsushita M, Li ST, Tsutsui K, Hisanaga S, Mikoshiba K, Takei K and Matsui H. Cophosphorylation of amphiphysin I and dynamin I by Cdk5 regulates clathrin-mediated endocytosis of synaptic vesicles. J Cell Biol 2003;163:813-824.

Tran PB and Miller RJ. Aggregates in neurodegenerative disease: crowds and power? Trends Neurosci 1999;22:194-197.

Tsai LH, Takahashi T, Caviness VS, Jr. and Harlow E. Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. Development 1993;119:1029-1040.

Tsai LH, Delalle I, Caviness VS, Jr., Chae T and Harlow E. p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. Nature 1994;371:419-423.

Tseng HC, Zhou Y, Shen Y and Tsai LH. A survey of Cdk5 activator p35 and p25 levels in Alzheimer's disease brains. FEBS Lett 2002;523:58-62.

Uchida T, Ishiguro K, Ohnuma J, Takamatsu M, Yonekura S and Imahori K. Precursor of cdk5 activator, the 23 kDa subunit of tau protein kinase II: its sequence and developmental change in brain. FEBS Lett 1994;355:35-40.

Wada Y, Ishiguro K, Itoh TJ, Uchida T, Hotani H, Saito T, Kishimoto T and Hisanaga S. Microtubule-stimulated phosphorylation of tau at Ser202 and Thr205 by cdk5 decreases its microtubule nucleation activity. J Biochem (Tokyo) 1998;124:738-746.

Van den Haute C, Spittaels K, Van Dorpe J, Lasrado R, Vandezande K, Laenen I, Geerts H and Van Leuven F. Coexpression of human cdk5 and its activator p35 with human protein tau in neurons in brain of triple transgenic mice. Neurobiol Dis 2001;8:32-44.

van Swieten JC, Rosso SM, van Herpen E, Kamphorst W, Ravid R and Heutink P. Phenotypic variation in frontotemporal dementia and parkinsonism linked to chromosome 17. Dement Geriatr Cogn Disord 2004;17:261-264.

Wang J, Liu S, Fu Y, Wang JH and Lu Y. Cdk5 activation induces hippocampal CA1 cell death by directly phosphorylating NMDA receptors. Nat Neurosci 2003;6:1039-1047.

Wang KK. Calpain and caspase: can you tell the difference? Trends Neurosci 2000;23:20-26.

Vartiainen N, Keksa-Goldsteine V, Goldsteins G and Koistinaho J. Aspirin provides cyclin-dependent kinase 5-dependent protection against subsequent hypoxia/reoxygenation damage in culture. J Neurochem 2002;82:329-335.

Weishaupt JH, Kussmaul L, Grotsch P, Heckel A, Rohde G, Romig H, Bahr M and Gillardon F. Inhibition of CDK5 is protective in necrotic and apoptotic paradigms of neuronal cell death and prevents mitochondrial dysfunction. Mol Cell Neurosci 2003;24:489-502.

Wen Y, Yang S, Liu R, Brun-Zinkernagel AM, Koulen P and Simpkins JW. Transient cerebral ischemia induces aberrant neuronal cell cycle re-entry and Alzheimer's disease-like tauopathy in female rats. J Biol Chem 2004;279:22684-22692.

Vignes M. Regulation of spontaneous inhibitory synaptic transmission by endogenous glutamate via non-NMDA receptors in cultured rat hippocampal neurons. Neuropharmacology 2001;40:737-748.

Wood JD, Beaujeux TP and Shaw PJ. Protein aggregation in motor neurone disorders. Neuropathol Appl Neurobiol 2003;29:529-545.

Wu HY, Tomizawa K, Oda Y, Wei FY, Lu YF, Matsushita M, Li ST, Moriwaki A and Matsui H. Critical role of calpain-mediated cleavage of calcineurin in excitotoxic neurodegeneration. J Biol Chem 2004;279:4929-4940.

Xie Z, Sanada K, Samuels BA, Shih H and Tsai LH. Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization, nuclear movement, and neuronal migration. Cell 2003;114:469-482.

Xiong W, Pestell R and Rosner MR. Role of cyclins in neuronal differentiation of immortalized hippocampal cells. Mol Cell Biol 1997;17:6585-6597.

Yamaguchi H, Ishiguro K, Uchida T, Takashima A, Lemere CA and Imahori K. Preferential labeling of Alzheimer neurofibrillary tangles with antisera for tau protein kinase (TPK) I/glycogen synthase kinase-3 beta and cyclin-dependent kinase 5, a component of TPK II. Acta Neuropathol (Berl) 1996;92:232-241.

Yang DD, Kuan CY, Whitmarsh AJ, Rincon M, Zheng TS, Davis RJ, Rakic P and Flavell RA. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature 1997;389:865-870.

Yoo BC and Lubec G. p25 protein in neurodegeneration. Nature 2001;411:763-764.

Yuan J, Lipinski M and Degterev A. Diversity in the mechanisms of neuronal cell death. Neuron 2003;40:401-413.

Zakeri Z and Lockshin RA. Cell death during development. J Immunol Methods 2002;265:3-20.

Zambrano CA, Egana JT, Nunez MT, Maccioni RB and Gonzalez-Billault C. Oxidative stress promotes tau dephosphorylation in neuronal cells: the roles of cdk5 and PP1. Free Radic Biol Med 2004;36:1393-1402.

Zhang BF, Peng FF, Zhang W, Shen H, Wu SB and Wu DC. Involvement of cyclin dependent kinase 5 and its activator p35 in staurosporine-induced apoptosis of cortical neurons. Acta Pharmacol Sin 2004a;25:1105-1111.

Zhang J, Krishnamurthy PK and Johnson GV. Cdk5 phosphorylates p53 and regulates its activity. J Neurochem 2002;81:307-313.

Zhang M, Li J, Chakrabarty P, Bu B and Vincent I. Cyclin-dependent kinase inhibitors attenuate protein hyperphosphorylation, cytoskeletal lesion formation, and motor defects in Niemann-Pick Type C mice. Am J Pathol 2004b;165:843-853.

Zhang Q, Ahuja HS, Zakeri ZF and Wolgemuth DJ. Cyclin-dependent kinase 5 is associated with apoptotic cell death during development and tissue remodeling. Dev Biol 1997;183:222-233.

Zheng M, Leung CL and Liem RK. Region-specific expression of cyclin-dependent kinase 5 (cdk5) and its activators, p35 and p39, in the developing and adult rat central nervous system. J Neurobiol 1998;35:141-159.

Zheng YL, Kesavapany S, Gravell M, Hamilton RS, Schubert M, Amin N, Albers W, Grant P and Pant HC. A Cdk5 inhibitory peptide reduces tau hyperphosphorylation and apoptosis in neurons. EMBO J 2005;24:209-220.

Zipfel GJ, Babcock DJ, Lee JM and Choi DW. Neuronal apoptosis after CNS injury: the roles of glutamate and calcium. J Neurotrauma 2000;17:857-869.

Zukerberg LR, Patrick GN, Nikolic M, Humbert S, Wu CL, Lanier LM, Gertler FB, Vidal M, Van Etten RA and Tsai LH. Cables links Cdk5 and c-Abl and facilitates Cdk5 tyrosine phosphorylation, kinase upregulation, and neurite outgrowth. Neuron 2000;26:633-646.

APPENDIX: ORIGINAL PUBLICATIONS (I-IV)

I

The levels of cdk5 and p35 proteins and tau phosphorylation are reduced during neuronal apoptosis

Kerokoski P, Suuronen T, Salminen A, Soininen H, Pirttilä T

Biochemical and Biophysical Research Communications 2001, 280: 998-1002.

II

Cleavage of the cyclin-dependent kinase 5 (cdk5) activator p35 to p25 does not induce tau hyperphosphorylation

Kerokoski P, Suuronen T, Salminen A, Soininen H, Pirttilä T

Biochemical and Biophysical Research Communications 2002, 298: 693-698.

III

Both N-methyl-D-aspartate (NMDA) and non-NMDA receptors mediate glutamate-induced cleavage of the cyclin-dependent kinase 5 (cdk5) activator p35 in cultured rat hippocampal neurons

Kerokoski P, Suuronen T, Salminen A, Soininen H, Pirttilä T

Neuroscience Letters 2004, 368: 181-185.

IV

Influence of phosphorylation of p35, an activator of cyclin-dependent kinase 5 (cdk5), on the proteolysis of p35

Kerokoski P, Suuronen T, Salminen A, Soininen H, Pirttilä T

Molecular Brain Research 2002, 106: 50-56.

PUBLICATIONS

SERIES OF REPORTS, DEPARTMENT OF NEUROLOGY

1. **Juhani Partanen (1978):** Time-locked phenomena of human motor unit potentials. An electromyographic study of satellites and doubles.
2. **Eeva Leino (1981):** Clinical and biochemical studies on progressive myoclonus epilepsy.
3. **Hilkka Soininen (1981):** Senile dementia. A clinical, neurochemical and etiological study.
4. **Rolf Danner (1982):** Adverse effects of anticonvulsive treatment on peripheral nerve conduction and posterior dominant EEG rhythm.
5. **Markku Saksa (1982):** The autonomic nervous system in experimental allergic neuritis. A functional, morphological and biochemical study.
6. **Juhani Sivenius (1982):** Studies on the rehabilitation, epidemiology and clinical features of stroke in East Central Finland.
7. **Asla Pitkänen (1987):** Somatostatin in epilepsy. An experimental and clinical study.
8. **Esa Mervaala (1987):** Evoked potential in human epilepsy. A neurophysiological study.
9. **Kari Reinikainen (1988):** Neurotransmitters in Alzheimer's disease.
10. **Tapani Keränen (1988):** Epilepsy in adults. An epidemiologic study in Eastern Finland.
11. **Jukka Jolkkonen (1988):** Vasopressin in the central nervous system. A study based on cerebrospinal fluid measurements.
12. **Jouni Sirviö (1989):** The cholinergic system in ageing and dementia. With special reference to acetylcholinesterase.
13. **Hannu Koponen (1989):** Delirium in the elderly. A clinical, neurochemical, neuropsychological and neuroradiological study.
14. **Asla Pitkänen (1989):** Somatostatin in experimental and human epilepsy.
15. **Eeva-Liisa Helkala (1990):** Memory in patients with Alzheimer's disease and demented patients with Parkinson's disease.
16. **-**
17. **Paavo Riekkinen Jr (1990):** Animal models of age-related degeneration of subcortical regulatory systems. With special reference to cholinergic, noradrenergic and serotonergic systems.
18. **Toivo Halonen (1990):** Neurotransmitter amino acids in epileptic convulsions and during vigabatrin treatment.
19. **Ulla Lepola (1990):** Panic disorder. A clinical, neurochemical, neuropsychological, and neuroradiological study.
20. **Kari Murros (1991):** Stress reactions of brain infarction. A prospective study on 105 patients with acute ischemic brain infarction of internal carotid artery territory.
21. **Aarne Ylinen (1991):** Hippocampal reactions and their pharmacotherapy in experimental epilepsy.
22. **Antti Valjakka (1992):** The subcortical deafferentation of the hippocampus and noradrenergic lesions as experimental models of dementia. Hippocampal electrophysiology.
23. **Aimo Rissanen (1992):** Cerebrovascular disease in the Jyväskylä region, Central Finland.
24. **Reetta Kälviäinen (1992):** Newly diagnosed epileptic seizure disorder in adults. A prospective follow-up study on 100 patients.
25. **Maria Mazurkiewicz (1992):** The effects of the enhanced GABAergic transmission on cognitive functions: An experimental study.
26. **Pekka Jäkälä (1992):** Modulation of attention and working memory by noradrenergic, serotonergic and cholinergic systems. An experimental neuropsychopharmacological study.
27. **Kari Alhainen (1992):** Anticholinesterase drug, tacrine (THA), in Alzheimer's disease. Discrimination of responders and nonresponders.
28. **Riitta Miettinen (1993):** Inhibitory circuits and subcortical innervation of the rat hippocampus: Implications for normal function and pathophysiological processes.
29. **Hannele Lahtinen (1993):** Hippocampus in experimental models of temporal lobe epilepsy. Amino acid-mediated neurotransmission and nerve cell injury following the transection of fimbria-fornix and the electrical stimulation of perforant pathway in rat.

30. **Päivi Hartikainen (1994):** Normal ageing. A neurochemical, neurophysiological and neuropsychological study with special reference to Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis.
31. **Outi Heinonen (1994):** Neuropathologic and peripheral markers of Alzheimer's disease with special emphasis on β-amyloid accumulation.
32. **Minna Riekkinen (1994):** The interactions between cholinergic and serotonergic systems in the modulation of spatial navigation and passive avoidance behavior. An experimental neuropsychopharmacological study.
33. **Keijo Koivisto (1995):** Population-based dementia screening program in the city of Kuopio, Eastern Finland: Evaluation of screening methods, prevalence of dementia and dementia subtypes.
34. **Arja Tuunainen (1995):** Evaluation of epileptic patients for temporal lobe surgery and postoperative follow-up. An electrophysiological study with neuropsychological, psychiatric and clinical correlates.
35. **Mervi Pitkänen (1995):** The role and pharmacological modulation of the NMDA receptor/channel on hippocampal synaptic transmission and behavior.
36. **Olli Kosunen (1996):** A neuropathologic study on Alzheimer's disease with a special emphasis on diagnostic accuracy.
37. **Mikko Laakso (1996):** MRI of hippocampus in incipient Alzheimer's disease.
38. **Maarit Lehtovirta (1996):** Familial Alzheimer's disease. A clinical and molecular genetic study.
39. **Tuomo Hänninen (1996):** Age-associated memory impairment. A neuropsychological and epidemiological study.
40. **Vesa Savander (1997):** Organization of intrinsic connections in the rat amygdaloid complex with special emphasis on the lateral, basal and accessory basal nuclei.
41. **Heikki Sorvari (1997):** Neurons containing calcium-binding proteins in the human amygdaloid complex.
42. **Tiina Kotti (1997):** Excitotoxicity-induced neuropathological changes in the rodent hippocampus. Possible functional consequences and drug treatments.
43. **Sirja Ruotsalainen (1997):** Serotonergic system and its interactions with cholinergic receptor mediated mechanisms in the modulation of working memory. An experimental study.
44. **Seppo Helisalmi (1998):** Molecular genetics of Alzheimer's disease with special emphasis on presenilin, amyloid beta precursor protein and apolipoprotein E genes.
45. **Merja Hallikainen (1998):** Age-associated memory impairment, and apolipoprotein E. A population-based clinical, neuropsychological, neurophysiological and neuroimaging study.
46. **Matti Vanhanen (1998):** Cognitive function in glucose intolerance in the elderly: the role of hyperinsulinemia.
47. **Kirsi Juottonen (1998):** MRI-volumes of the entorhinal, perirhinal and temporopolar cortices in normal aging and in Alzheimer´s disease.
48. **Raimo Pussinen (1999):** An experimental study on the role of α_1-adrenoceptors and putrescine in the modulation of hippocampal plasticity and memory encoding - interactions with NMDA receptors.
49. **Tarja Puumala (1999):** Monoamines in the modulation of attention and response inhibition: development of a new animal model of attention deficit and impulsivity.
50. **Mia Mikkonen (1999):** The human entorhinal cortex. Anatomic organization and its alteration in Alzheimer's disease and temporal lobe epilepsy.
51. **Jukka Puoliväli (2000):** An experimental study on the cholinergic modulation of cortical arousal and cognitive functions. With special emphasis on apolipoprotein E.
52. **Kauko Pitkänen (2000):** Stroke rehabilitation in the elderly. A controlled study of the effectiveness and costs of a multidimensional intervention.
53. **Mikko Hiltunen (2000):** A molecular genetic study of factors involved in Alzheimer's disease.
54. **Sami Ikonen (2001):** The role of the septohippocampal cholinergic system in cognitive functions.
55. **Tuuli Salmenperä (2001):** Damage in the hippocampus, amygdala, entorhinal and perirhinal cortex of adults with partial epilepsy.
56. **Zinayida Bezvenyuk (2001):** Multiple pathways of DNA disintegration during neuronal apoptosis.

57. **Tero Tapiola (2001):** Biological markers for Alzheimer's disease. With special emphasis on cerebrospinal fluid ß-amyloid and tau.
58. **Kirsi Puurunen (2001):** The effects of pharmacotherapy and training on functional recovery after global and focal cerebral ischemia in rats.
59. **Maaria Ikonen (2001):** Apoptosis-associated changes in neuronal gene expression. With special emphasis on the insulin-like growth factor system.
60. **Inga Kadish (2002):** Plasticity in the entorhinal-hippocampal pathway. Influences of gene mutations and hormones.
61. **Pauliina Korhonen (2002):** Gene regulation in neuronal degeneration - Role of mSin3 and YY1 factors.
62. **Miia Kivipelto (2002):** Vascular risk factors in Alzheimer's disease and mild cognitive impairment. A longitudinal, population-based study.
63. **Margit Overmyer (2002):** Gliosis in relation to Alzheimer's hallmark lesions in aging and Alzheimer's disease. A postmortem immunohistochemical study.
64. **Marja Äikiä (2002):** Verbal memory in newly diagnosed partial epilepsy. A neuropsychological study.
65. **Li Liu (2003):** Cholinergic neurotransmission, amyloid-β peptide and the pathogenesis of Alzheimer's Disease. A study in the APP and PS1 double transgenic mouse model.
66. **Jun Wang (2003):** The role of Aβ-peptide on spatial memory, EEG, auditory evoked potentials and nicotinic cholinergic receptors in A/P transgenic mice.
67. **Juhana Aura (2003):** Interaction of muscarinic acetylcholine and N-methyl-D-aspartate –type glutamate receptors in the regulation of spatial learning and memory.
68. **Johanna Kuhmonen (2003):** Neuroprotection in experimental acute cerebral ischaemia: α2-adrenoreceptor agonism, MAO-B inhibition, and enhancement of GABAergic neurotransmission as neuroprotective strategies.
69. **Jaana Autere (2003):** Genetics of Parkinson's Disease in the Finnish Population.
70. **Erkki Kuusisto (2004):** Role of the p62 protein in the formation of neuropathological cytoplasmic inclusions.
71. **Maija Pihlajamäki (2004):** Functional MRI studies on human declarative memory.
72. **Chuan-sheng Zhao (2005):** Psychotropic medication and functional recovery following cortical stroke in aged rats.
73. **Dimitrije Jakovljević (2005):** The roles of chronobiological and socioeconomic factors in the occurrence of cerebrovascular diseases.
74. **Sinikka Peurala (2005):** Rehabilitation of gait in chronic stroke patients.
75. **Laura Parkkinen (2005):** Impact of α-synuclein pathology on aging.
76. **Iain Wilson (2005):** Hippocampal place cells as a window into cognitive aging.
77. **Susan Iivonen (2005):** Genetic and expressional studies of Alzheimer's disease candidate genes. Emphasis on CYP19, seladin-1 and HSPG2 genes.
78. **Jouni Ihalainen (2005):** Regulation of dopamine release in the forebrain by alpha2-adrenoceptors and NMDA glutamate receptors - a microdialysis study.
79. **Giedrius Kalesnykas (2005):** Cholinergic neurons of the rodent basal forebrain and their content of estrogen receptor alpha.
80. **Marina Boccardi (2006):** MRI studies in frontotemporal dementia.
81. **Anne Koivisto (2006):** Genetic components of late-onset Alzheimer's disease with special emphasis on ApoE, IL-6, CYP46, SERPINA3 and PPARγ.
82. **Taneli Heikkinen (2006):** Cognitive effects of estrogen in ovariectomized, aged and transgenic mice modelling Alzheimer's disease.
83. **Minna Korolainen (2006):** Proteomic analysis of post-translationally modified proteins in Alzheimer's disease.